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Initiation and Regulation of Type 2 Immunity and Inflammation at Mucosal Sites

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Abstract
Identifying the cellular and molecular requirements for initiating and regulating type 2 immunity and inflammation is essential for the development of new vaccines against helminth parasites and treatments for atopic diseases. CD11c+ dendritic cells (DCs) are critical antigen-presenting cells (APCs) capable of priming and promoting the differentiation of naïve CD4+ T cells. However, the role of DCs in the initiation of Th2 cell differentiation following exposure to helminth parasites and allergens remains unclear. In Chapter 2, we examine the cellular requisites for initiating Th2 cytokine-dependent immunity and inflammation in the gastrointestinal tract utilizing infection with the helminth Trichuris muris. By genetic restriction of MHC class II expression to CD11c+ DCs we demonstrate that, in contrast to Th1-cytokine-mediated immunity, antigen presentation by CD11c+ cells is insufficient to generate protective type 2 immune responses in vivo, suggesting additional non-DC APC interactions may be required for Th2 cell differentiation. In Chapter 3 we identify basophils as a cell population that expanded following exposure to Trichuris and expressed both IL-4 message and MHC class II. Depletion of basophils resulted in impaired immunity to Trichuris and purified basophils promoted CD4+ T cell proliferation and Th2 cell differentiation in vitro and in vivo. Chapter 4 explores the regulation of type 2 immune responses in the intestine and lung by IL-31, a cytokine produced predominantly by Th2 cells that signals through a heterodimeric receptor composed of OSMR and IL-31Rα. IL-31Rα deficient mice exhibited enhanced Th2 cytokine production, elevated IgE levels and increased type 2 inflammation following both Trichuris infection and in an acute model of airway inflammation after exposure to Schistosoma mansoni eggs. Taken together, the results presented in this thesis demonstrate that DCs are not sufficient to promote Th2 cell responses in vivo during Trichuris infection and are the first report of an APC function for basophils in promoting Th2 cell responses. In addition, they identify a novel regulatory role for IL-31R in limiting the magnitude of Th2 cytokine-dependent immunity and inflammation.

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Mom, this one's for you.

ABSTRACT

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Jacqueline Gage Perrigoue

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Identifying the cellular and molecular requirements for initiating and regulating type 2 immunity and inflammation is essential for the development of new vaccines against helminth parasites and treatments for atopic diseases. $CD11c^+$ dendritic cells (DCs) are critical antigen-presenting cells (APCs) capable of priming and promoting the differentiation of naïve $CD4^+$ T cells. However, the role of DCs in the initiation of Th2 cell differentiation following exposure to helminth parasites and allergens remains unclear. In Chapter 2, we examine the cellular requisites for initiating Th2 cytokine-dependent immunity and inflammation in the gastrointestinal tract utilizing infection with the helminth *Trichuris muris*. By genetic restriction of MHC class II expression to $CD11c^+$ DCs we demonstrate that, in contrast to Th1-cytokine-mediated immunity, antigen presentation by $CD11c^+$ cells is insufficient to generate protective type 2 immune responses *in vivo*, suggesting additional non-DC APC interactions may be required for Th2 cell differentiation. In Chapter 3 we identify basophils as a cell population that expanded following exposure to *Trichuris* and expressed both IL-4 message and MHC class II. Depletion of basophils resulted in impaired immunity to *Trichuris* and purified basophils promoted $CD4^+$ T cell proliferation and Th2 cell differentiation *in vitro* and *in vivo*. Chapter 4 explores the regulation of type 2 immune responses in the intestine and lung

by IL-31, a cytokine produced predominantly by Th2 cells that signals through a heterodimeric receptor composed of OSMR and IL-31Rα. IL-31Rα deficient mice exhibited enhanced Th2 cytokine production, elevated IgE levels and increased type 2 inflammation following both *Trichuris* infection and in an acute model of airway inflammation after exposure to *Schistosoma mansoni* eggs. Taken together, the results presented in this thesis demonstrate that DCs are not sufficient to promote Th2 cell responses *in vivo* during *Trichuris* infection and are the first report of an APC function for basophils in promoting Th2 cell responses. In addition, they identify a novel regulatory role for IL-31R in limiting the magnitude of Th2 cytokine-dependent immunity and inflammation.

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Publications

The contents of this thesis include portions of modified text and figures from the following published manuscripts:

Perrigoue JG, Li J, Zaph C, Goldschmidt M, Scott P, de Sauvage FJ, Pearce EJ, Ghilardi N, Artis D. "IL-31-IL-31R interactions negatively regulate type 2 inflammation in the lung." J Exp Med. Mar 2007; 204(3):481-7.

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Perrigoue JG, Zaph C, Guild K, Du Y, and Artis D. "IL-31-IL-31R interactions limit the magnitude of Th2 cytokine-dependent immunity following intestinal helminth infection." J Immunol. May 2009; 182(10):6088-94.

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Chapter 1
Introduction

The barrier surfaces of the body, including the skin, oral and nasal cavities, respiratory tract, female genital tract and gastrointestinal tract, are the major sites at which we are exposed to both environmental antigens and pathogens. Of these sites, the intestine is faced with the unique challenge of juxtaposing the largest repository of immune cells in the body with arguably the largest depot of foreign antigen including ingested environmental material, food products, nearly 10^{14} commensal bacteria and numerous viral and parasitic microorganisms. The capacity to rapidly respond to potential invading pathogens must be balanced with the need to maintain tolerance to both environmental antigens and beneficial commensal microorganisms. Insufficient responses to pathogens could result in susceptibility to infection while excessive inflammatory immune responses may lead to a loss of barrier integrity with potentially pathological consequences ranging from food allergy and colitis to sepsis. Understanding the mechanisms responsible for initiating and regulating intestinal immune responses is therefore essential for the design of effective oral vaccines as well as identifying potential therapeutic targets for multiple allergic and inflammatory diseases.

This thesis will explore novel mechanisms involved in the development and regulation of mucosal immune responses, with an emphasis on $CD4^+$ T helper type 2 (Th2) cytokine-dependent immunity and inflammation associated with helminth infection and atopic disease. In **Chapters 2 and 3**, the cellular requirements for antigen presentation in the development of type 2 immune responses will be investigated while the regulation of

type 2 immune responses by the cytokine interleukin (IL)-31 will be assessed in **Chapter 4**. Finally, the functional consequences of intestinal epithelial cell (IEC)-intrinsic MHC class II expression in the steady state and during enteric infection will be interrogated in **Appendix A**.

This chapter will serve as an introduction to the gastrointestinal tract, the development and function of $CD4^+$ helper T cell subsets, how helminth parasites influence the initiation of Th2 cell responses, the use of *Trichuris muris* infection as a model for Th2 cytokine-dependent immunity in the intestine and the gp130 family of cytokines and cytokine receptors.

1.1 Immunity in the gastrointestinal (GI) tract

Throughout this thesis, I will utilize *in vivo* models of infection or inflammation to investigate the development and regulation of $CD4^+$ Th2 cell-dependent immune responses at mucosal sites. Here, I will introduce the physiology of the intestinal immune system, including the structure of intestinal lymphoid tissues, current models of antigen sampling and adaptive immune responses to oral antigen.

1.1.1 Organization of the gut-associated lymphoid tissue (GALT)

Beneath the single cell layer of epithelial cells that separates the luminal contents of the gut from the underlying tissue, there are a wide array of lymphoid aggregates and organized secondary lymphoid structures including cryptopatches (CPs), isolated lymphoid follicles (ILFs), Peyer's patches and mesenteric lymph nodes (mLNs) (**Fig. 1**). CPs, the least organized and perhaps least understood of intestinal lymphoid tissues, are aggregates of approximately 10^3 lineage negative (lin^-), c-kit^+ IL-$7R^+$ cells surrounded

by CD11c^{+} dendritic cells (DCs) and VCAM-1^{+} stromal cells that are located at the base of intestinal crypts throughout the small and large intestine (Kanamori *et al.*, 1996). While the requisites for CP formation including the γt isoform of retinoid acid-related orphan receptor (RORγt), IL-7R, lymphotoxin (LT) and lymphotoxin beta receptor (LTβR) have been identified, their function remains largely unclear (Colonna, 2009; Newberry and Lorenz, 2005). Early reports demonstrated that adoptive transfer of c-kit^{+} but not c-kit^{-} CP cells into irradiated SCID recipients resulted in the reconstitution of T cells within the intraepithelial lymphocyte (IEL) compartment and mLNs and suggested that CPs were a source of hematopoietic progenitor cells that could support extrathymic intestinal T cell development (Saito *et al.*, 1998). Supporting this observation CPs, as well as IELs, are present in athymic (*nu/nu*) mice (Kanamori *et al.*, 1996). Recent data however has contradicted these studies and an alternative hypothesis for CP function was proposed. In a series of cell fate mapping experiments using transgenic reporter mice, Littman and colleagues demonstrated that IELs were still present in the absence of both RORγt and CP cells, and that CD4-driven eGFP expression was readily detected in the thymus but never within CPs (Eberl and Littman, 2004). These data suggested that CP cells were unlikely to be a source of T cell progenitors and that TCRαβ IELs were of thymic origin. Based on similarities in surface phenotype, the lin^{-}c-kit^{+}IL-7R^{+} cells within CPs were proposed to be analogous to the fetal lymphoid-tissue inducer (LTi) cells that are responsible for organizing lymph nodes and Peyer's patches and as such may serve to organize secondary lymphoid tissues within the intestine rather than support extrathymic T cell development (Eberl and Littman, 2004).

ILFs and Peyer's patches are closely related lymphoid structures, composed largely of conventional B-2 B cells, that contain a loosely organized germinal center with a follicle-associated epithelium (FAE) (Newberry and Lorenz, 2005). Within the FAE are specialized epithelial cells called microfold (M) cells (Owen and Jones, 1974) that function to actively transport antigens across the intestinal barrier for uptake by professional antigen presenting cells (APCs) in the lamina propria. It is important to note that while ILFs can be found throughout the small and large intestine, Peyer's patches are present only in the small intestine and these clusters of lymphoid follicles have a more defined structure than ILFs. Below the FAE of Peyer's patches is an area called the subepithelial dome that contains an extensive network of DCs that are thought to aquire antigen from M cells and prime naïve T cell responses locally. It has also been proposed that these DCs could traffic to the mLNs to deliver antigen derived from Peyer's patches or directly interact with mLN T cells. However a recent study examining the composition of DCs in pigs found that the phenotype of migratory DCs in the mLNs more closely resembles lamina propria DCs than Peyer's patch DCs (Bimczok *et al.*, 2005). While contributing to the function of ILFs and Peyer's patches as primary inductive sites for adaptive immune responses within the intestine, the specialization of M cells within the FAE for rapid transcytosis of luminal material also provides an ideal pathway that many enteric pathogens, perhaps most notably *Salmonella typhimurium,* utilize to gain access to host tissue (Corr *et al.*, 2008). Exploitation of M cell-mediated entry into the intestine however can also be used to inform the design of both oral and nasal vaccines and this strategy of targeting antigens for uptake by M cells to improve mucosal immune responses has been employed by a number of groups in recent years (Chionh *et al.*, 2009; Nochi *et al.*, 2007; Wu *et al.*, 2001).

The mLNs are the primary draining lymph nodes serving the intestinal tissue and their structure is similar to other peripheral lymph nodes (pLNs) throughout the body. However, in comparison to other pLNs, the mLNs possess unique functional capacities including the imprinting of gut-homing properties to lymphocytes and the induction of oral tolerance. For example, a technically elegant set of experiments using reciprocal surgical transfer of pLNs into the location of mLNs recently demonstrated that the stromal cells within the mLNs are required for imprinting expression of the gut-homing integrin $\alpha4\beta7$ and the chemokine receptor CCR9 to lymphocytes and that splenic DCs, that can not induce expression of gut-homing molecules to T cells *in vitro*, are able to do so *in vivo* following direct injection into the afferent mLN lymphatics (Hammerschmidt *et al.*, 2008). This capacity to imprint gut-homing receptors was found to be partially mediated through selective expression of retinoic acid (RA)-producing enzymes RALDH1 and RALDH3 within mLN stroma compared to pLN stroma and suggests that non-hematopoietic cells within the mLN, perhaps in cooperation with migratory lamina propria DCs, are uniquely capable of imprinting lymphocytes with a gut-homing phenotype following priming (Hammerschmidt *et al.*, 2008). Similar to ILFs and Peyer's patches, mLNs are also considered to be inductive sites for intestinal adaptive immune responses including the tolerization and priming of T cells and T cell-dependent B cell IgA class switch recombination (Cerutti, 2008). The role of mLNs in the generation of oral tolerance and the potential pathways of T helper cell differentiation after antigen-specific priming will be discussed in further detail in sections **1.1.3** and **1.2**.

1.1.2 Antigen sampling in the GI tract

The microenvironment of the GI tract poses a significant challenge to the development of an adaptive immune response in that the necessity for mucosal integrity requires a functional barrier between luminal antigens and intestinal lymphocytes. This barrier is created by physical impediments including IEC tight junctions, the filamentous brush border glycocalyx and secretion of mucins in addition to molecular impediments including anti-microbial peptides and secretory IgA (Nagler-Anderson, 2001). Notwithstanding this, it is clear that pathogen-derived antigens gain access to accessory cells, although the pathways through which this occurs are not completely understood. The primary pathway for antigen sampling in the intestine is considered to be trans-epithelial vesicular transport by M cells (Kraehenbuhl and Neutra, 2000). As discussed above, M cells are located within the FAE that overlies organized intestinal lymphoid tissues and were also recently identified in non-follicle associated epithelium of the small intestine (Jang *et al.*, 2004), however whether these isolated M cells exist in the colon is unknown. Antigen delivered by M cells is taken up by DCs within the sub-epithelial dome of Peyer's patches that can then migrate to T cell areas to prime naïve T cell responses (**Fig. 2a**). The fate of antigen transcytosed by M cells outside of the Peyer's patches is not known but it is likely that DCs or other APCs within the lamina propria could aquire this antigen and migrate to the mLNs to prime naïve T cell responses. Another mechanism for antigen sampling in the intestine is the penetration of epithelial cell tight junctions by the processes of a subset of $CD103^+$ CX_3CR1^+ lamina propria DCs that are then able to directly sample lumenal bacteria (Chieppa *et al.*, 2006; Niess *et al.*, 2005; Rescigno *et al.*, 2001) (**Fig. 2b**). However, this mechanism of sampling luminal contents appears to be limited to a small portion of the small intestine and may be strain-

dependent as these projections have only been reported in C57BL/6 mice (Vallon-Eberhard *et al.*, 2006). In addition to active transport by M cells, IEC-specific expression of an MHC class I-like Ig receptor, the neonatal Fc receptor (FcRn) (Simister and Mostov, 1989), allows for receptor-mediated sampling of luminal antigens for delivery to lamina propria APCs (**Fig. 2c**) and has recently been shown to contribute to recognition of the cecal-dwelling bacterial pathogen *Citrobacter rodentium* in a transgenic mouse model (Yoshida *et al.*, 2006).

While all of the above mechanisms involve uptake of antigen by lamina propria DCs for presentation to T cells, IECs themselves have been shown to express the molecular machinery required for antigen processing and presentation via MHC class II and may directly, or indirectly via peptide-loaded MHC class II in exosomes (Mallegol *et al.*, 2005), influence intestinal $CD4^+$ T cell responses (**Fig. 2d**). Early *in vitro* studies using epithelial cells isolated from rat and murine small intestine demonstrated that IECs, while significantly less potent than professional antigen presenting cells, are capable of both processing and presenting antigen via the MHC class II pathway (Telega *et al.*, 2000; Kaiserlian, 1999; Kaiserlian *et al.*, 1989; Bland and Warren, 1986b; Bland and Warren, 1986a). In support of an *in vivo* role for MHC class II presentation by IEC, a study in Crohn's disease patients has shown that MHC class II expression is dramatically upregulated in IECs of diseased colonic tissue compared to healthy tissue and that soluble ovalbumin (OVA) protein administered topically during colon biopsy is efficiently taken up by IEC and localized to late endosomes on their basolateral surface (Buning *et al.*, 2006). The reported lack of detection of co-stimulatory molecule expression on IECs (Sanderson *et al.*, 1993) suggests that in contrast to predicted roles in priming T cell

responses and promoting intestinal inflammation, IEC-intrinsic MHC class II expression may be involved in the initation or maintenance of oral tolerance (Mowat, 2003). The functional consequences of IEC-intrinsic MHC class II expression however are unknown and will be explored in **Appendix A** of this thesis.

1.1.3 Immune responses to enteric antigens

Antigen uptake at mucosal sites can result in the development of antigen-specific immunoregulatory or immunostimulatory responses. Determining the mechanisms by which innate immune cells within the intestine distinguish between innocuous and pathogen-derived antigens to promote the development of either tolerogenic or immunogenic responses is one of the fundamental questions in mucosal immunology.

The development of oral tolerance, or non-responsiveness to peripheral antigen stimulation subsequent to oral administration of that antigen, is well documented, however where and how adaptive tolerance is initiated has been an area of contention. The role Peyer's patches play in the induction of oral tolerance has been particularly unclear and there are data to suggest both that Peyer's patches are required for the induction of oral tolerance (Fujihashi *et al.*, 2001) and that in Peyer's patch-deficient mice (Spahn *et al.*, 2001) and within intestinal ligated loops devoid of Peyer's patches (Kraus *et al.*, 2005), tolerance is unperturbed. Current data support a model in which the mLNs are a critical site for induction of oral tolerance and mice subjected to mesenteric lymphadenectomy or mice that are deficient in CCR7, a molecule required for the migration of intestinal DCs to the mLNs, are rendered refractory to tolerization (Worbs *et al.*, 2006). Through cannulation of the lymph in rats, it has been suggested

that small numbers of migratory DCs within the lamina propria continually traffic to the mLNs in the steady state (Pugh *et al.*, 1983) with estimates of 800,000 DCs migrating to the mLNs each day (Milling *et al.*, 2007). Subsequent studies demonstrated that a subset of these migratory DCs can consitutively deliver both commensal bacteria (Macpherson and Uhr, 2004) and apoptotic IECs (Huang *et al.*, 2000) from the lamina propria to the mLNs. These data are consistent with a model whereby constitutive migration of intestinal DCs carrying commensal- or food-derived products as well as self-antigens from apopototic IECs to the mLNs may be involved in the promotion of oral tolerance.

In support of a central role for DCs in the induction of tolerance, several studies have demonstrated that subsets of DCs isolated from mucosal sites preferentially induce tolerogenic or type 2 $CD4^+$ T cell responses, including peripheral $Foxp3^+$ T_{reg} differentation and increased IL-10 production, compared to their splenic DC counterparts (Coombes *et al.*, 2007; Sun *et al.*, 2007; Iwasaki and Kelsall, 1999). This tolerogenic or anti-inflammatory DC phenotype can be replicated *in vitro* in both human and murine cells by culture of peripheral blood or bone marrow-derived DCs with supernatants from IEC lines. These "IEC-conditioned" DCs adopt a non-inflammatory phenotype with a reduced expression of IL-12 that is dependent upon IEC-derived thymic stromal lymphopoeitin (TSLP) (Rimoldi *et al.*, 2005) and the ablility to promote Foxp3 expression in $CD4^+$ T cells in the presence of TGFβ and RA (Iliev *et al.*, 2009), suggesting potential crosstalk between IECs and lamina propria DCs *in vivo*. Consistent with this hypothesis, NFκB-dependent expression of the IEC-derived TSLP is required *in vivo* to inhibit DC

production of IL-12 and to limit intestinal inflammation in murine models of colitis and enteric infection (Taylor *et al.*, 2009; Zaph *et al.*, 2007).

Besides indirectly influencing tolerance to oral antigen via conditioning of DCs, IECs themselves, as discussed above, may directly suppress local T cell responses via expression of MHC class II in the absence of co-stimulatory molecules. Alternatively, IECs may indirectly influence antigen-specific tolerance or immunity to orally encountered antigens by secretion of MHC class II-antigen complexes found within exosomes (Karlsson *et al.*, 2001; van Niel *et al.*, 2001). Exosomes secreted by IECs have been termed “tolerosomes” and have been used to transfer tolerance to the model antigen OVA to recipient mice in an MHC class II-dependent manner (Ostman *et al.*, 2005; Karlsson *et al.*, 2001). The hypothesis that IEC antigen presentation is involved in tolerance induction will be formally tested in **Appendix A** through the generation of mice with an IEC-specific deletion in MHC class II.

While in the steady state a tolerogenic, non-inflammatory immune response to luminal antigens appears to be the default pathway in healthy individuals, exposure to enteric pathogens requires an alternate program of innate immune cell recognition and activation to develop protective immunity. As in other peripheral tissues, activation of innate cells within the intestine is likely mediated through the recogntion of pathogen-associated molecular patterns (PAMPs) by germline-encoded pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), C-type lectins, the intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and cytosolic nucleic acid receptors such as RIG-1-like receptors (RLRs) and the cytosolic DNA

sensor, ISD (Palm and Medzhitov, 2009), although the relative importance of innate recognition by non-hematopoeitic or hematopoeitic cells is yet to be determined. Paradoxically, many of the same pathways that are activated in the intestinal epithelium following exposure to pathogenic bacteria are shared and even beneficial when activated by commensal flora. For example, the active basal or "tonic" stimulation of IEC by commensal flora via MyD88 and NFκB signaling pathways is required for the maintenance of intestinal homeostasis (Nenci *et al.*, 2007; Rakoff-Nahoum *et al.*, 2004). Discrimination between commensal flora and potentially pathogenic microorganisms at the mucosal barrier is therefore thought to be mediated by a number of mechanisms from selective expression of PRRs on the basal versus apical surface of IECs to active inhibitory signals delivered to IEC by commensal organisms (Artis, 2008).

Once pathogens gain access to the intestinal host tissue, whether through a physical breach in the epithelial barrier, uptake by M cells, or active invasion, activation of innate immune cells within the lamina propria is predicted to result in a local inflammatory response associated with the migration of intestinal DC subsets to T cell zones. For example, following exposure to soluble *Toxoplasma gondii* antigen (STAg) (Iwasaki and Kelsall, 2000) or infection with *Salmonella typhimurium* (Salazar-Gonzalez *et al.*, 2006) subepithelial dome Peyer's patch $CD11b^+$ DCs migrate into T cells zones within the FAE in a CCR6/MIP-3α-dependent manner. Additionally, systemic stimulation with bacterial components like LPS can dramatically increase the rate of migration of lamina propria DCs to the mLNs (Turnbull *et al.*, 2005). Migration of DCs to T cell areas following pathogen challenge can thus facilitate the priming of antigen-specific T cell responses and, depending on the context of DC-activation, promote the differentiation of $CD4^+$ T

cells into the appropriate specialized helper cell lineage (discussed in detail below, section **1.2**) to eliminate the offending pathogen.

1.2 Helper T cell differentiation

The development of protective adaptive immunity is dependent upon the activation of antigen-specific $CD4^+$ T cells by innate accessory cells through signals in *cis* and *trans*, including T cell receptor (TCR) engagement, co-stimulatory molecule activation, Notch signaling and autocrine and paracrine cytokine signaling, that converge to specify the appropriate helper T cell fate required to combat the invading pathogen (Reiner, 2008; Zhu and Paul, 2008). The ability of the resultant specialized helper T cells to mobilize discrete populations of innate as well as adaptive effector cells in response to disparate classes of pathogens is a central part of mammalian host defense. These effector helper cell lineages, T helper (Th) type 1, Th2 and Th17 cells as well as adaptive regulatory T cells (aTreg), are defined by their unique cytokine profiles, transcription factor expression and function (**Fig. 3**). In this section, I will introduce these subsets and discuss their functions in regulating immunity and inflammation, the molecular requisites for their development and the mechanisms by which innate immune cells promote Th cell differentiation.

1.2.1 Specialization of helper T cells in response to diverse pathogens

Two helper cell subsets, Th1 and Th2, were initially defined by their unique expression of signature cytokines including IFN-γ and IL-4 (or B cell stimulating factor-1, BSF1) and their differential effects on B cell responses (Mosmann *et al.*, 1986). However, the first evidence supporting a biological function for these helper T cell subsets in immunity

came from *in vivo* infection models. Protective immunity to the intracellular protozoan parasite *Leishmania major* for example was shown to be mediated by T cells of a Th1 phenotype and inhibited by Th2 cell clones (Scott *et al.*, 1988) and *in vivo* models of infection continue to shape our understanding of Th cell function and the requirements for Th cell differentiation in host defense.

Currently, there are three primary subsets of effector helper T cells, Th1, Th2 and Th17 cells associated with defense against pathogens while adaptive regulatory T cells (aTreg) provide counter-regulation to these effector responses to limit inflammation. Th1 cell responses, characterized by expression of IFN-γ, mediate immunity to pathogens such as viruses and intracellular parasites and bacteria while Th2 cells, through secretion of effector cytokines including IL-4, IL-5 and IL-13, are required for resistance to gastrointestinal helminth parasites. Th17 cells, the most recently described helper cell lineage, are associated with defense against certain extracellular bacteria and fungal pathogens and their signature cytokine profile includes IL-17A, IL-17F, IL-21 and IL-22. Although we are still in the early stages of determining the function of Th17 cells, the recent identification of human patients with defects in Th17 cell development support our current understanding of their protective role. T cells isolated from patients with hyper-IgE syndrome (HIES or Job's syndrome) are unable to produce IL-17 upon mitogenic stimulation or undergo Th17 cell differentiation *in vitro* and present with frequent staphylococcal and fungal infections, consistent with a protective role for IL-17 in defense against these classes of pathogens (Milner *et al.*, 2008).

These effector Th cell lineages are associated not only with protective immunity to pathogens, but also with the potential to drive immune-mediated pathology. Excessive Th1 cell and Th17 cell responses are associated with organ-specific autoimmune and inflammatory diseases including experimental autoimmune encephalitis (EAE), inflammatory bowel disease (IBD), psoriasis and arthritis. Analyses of mice deficient in unique molecular determinants of Th1 or Th17 cell fate however have facilitated identification of the non-redundant functions of these lineages in promoting disease, and in many cases Th17 cells have taken a more prominent role in driving autoimmune pathology. The progression of EAE in particular was shown to be independent of IL-12 but dependent on IL-23, via the promotion of IL-17-producing T cells, providing the first evidence that Th1 cells may not be the critical players in driving EAE pathology (Langrish *et al.*, 2005; Cua *et al.*, 2003). In experimental models however it is important to note that depending on the context in which disease is induced, the requirements for Th1 or Th17 cells to promote disease may differ. For example, in the case of experimental autoimmune uveitis, disease induction by immunization with retinal antigen in Freund's adjuvant was IL-17-driven, while induction by retinal antigen-pulsed dendritic cells or T cell transfer was dependent on IFN-γ (Luger *et al.*, 2008). In addition to their role in autoimmune inflammation, Th17 cell responses have also been linked to the promotion of tumor growth (Wang *et al.*, 2009) and a recent study showed that Th17 cells induced by a common human commensal bacterium resulted in IL-17-dependent colitis and colonic tumor formation in the *Min* mouse intestinal neoplasia model (Wu *et al.*, 2009), suggesting that IL-17 may be an important link between inflammation and cancer.

In the case of Th2 cells, the same effector functions that promote expulsion of helminths, including goblet cell hyperplasia, increased mucin production, eosinophilia and mast cell degranulation, are characteristics of allergic inflammation and asthma. Consistent with this conserved function for Th2 cells, in humans, polymorphisms associated with a predisposition to allergy are also linked to enhanced immunity to helminth parasites (Hopkin, 2009). Excessive Th2 cytokine production as a result of chronic helminth infection or chronic allergen exposure is also associated with progression to fibrotic disease and IL-13 signaling in particular has a strong causative association with fibrosis. Infection with the helminth *Schistosoma mansoni,* where accumulation of parasite eggs results in chronic type 2 inflammation, has provided important insights into the role of IL-13/IL-13R signaling in fibrotic disease and was used to identify IL-13 as a critical pro-fibrotic factor during chronic Th2 cytokine-mediated inflammation. IL-13 signals through a dimeric receptor composed of the IL-4Rα and IL-13Rα1 chains. However a second secreted "decoy" receptor, the soluble IL-13Rα2 chain, is upregulated following IL-4/IL-13 signaling and has been associated with the negative regulation of type 2 inflammation. For example, IL-13Rα2 is required for the inhibition of fibrosis in both *S. mansoni* infection (Chiaramonte *et al.*, 2003) and airway inflammation models (Wilson *et al.*, 2007) and treatment with soluble IL-13Rα2-Fc can inhibit IL-13-induced liver fibrosis (Chiaramonte *et al.*, 1999). There is also data however to suggest a pro-fibrotic role for a putative signaling form of IL-13Rα2 induced by IL-13 and TNFα. In these studies, signaling through IL-13Rα2 resulted in the induction of TGF-β and collagen deposition in an acute lung inflammation model (Fichtner-Feigl *et al.*, 2006) and was linked to the development of fibrosis in chronic colitis (Fichtner-Feigl *et al.*, 2007). These disparate results may reflect differences in STAT6-dependent versus TGF-β-dependent pathways

of IL-13-mediated fibrosis and underscore the challenges in studying these signaling pathways *in vivo.*

1.2.2 Molecular requirements for Th cell differentiation

Th1 cell differentiation involves two main signaling pathways, IL-12/STAT4 signaling and IFN-γ/STAT1 signaling, and expression of the master-regulator of Th1 cell differentiation, the T-box binding transcription factor T-bet. T-bet upregulation as a result of T cell activation is enhanced by IFN-γ/STAT1 signaling and contributes to Th1 cell differentiation through a number of pathways including the upregulation of IL-12Rβ2, allowing the selective expansion of T-bet-expressing cells via APC-derived IL-12, synergizing with other transcription factors to further induce IFN-γ expression and inhibiting IL-4 expression via binding of a silencer element within the IL-4 promoter (Amsen *et al.*, 2009). T-bet can also negatively regulate Th2 cell differentiation through a kinase-dependent interaction with GATA3 that interferes with GATA3 DNA-binding activity (Hwang *et al.*, 2005).

The molecular requirements for optimal Th2 cell differentiation include signaling via the IL-4/STAT6 and IL-2/STAT5 pathways and expression of the transcription factor GATA3. IL-4/STAT6 signaling in conjunction with TCR signaling results in the upregulation of GATA3 while the signaling via IL-2/STAT5 is thought to stabilize IL-4 expression and provide signals for the survival and expansion of Th2 cells. Early experiments looking for factors that drive Th2 cell differentiation identified the transcription factors NFAT, AP-1 and c-maf as synergistically contributing to IL-4 production through consensus binding sites within the IL-4 promoter (Ho *et al.*, 1996; Rooney *et al.*, 1995) but GATA3 alone

appears to be capable of inducing full transactivation of the IL-4 locus, including IL-5 and IL-13 expression, and reciprocal regulation of Th1 cell differentiation through inhibition of IL-12Rβ2 expression (Amsen *et al.*, 2009). Although use of recombinant IL-4 is the standard for *in vitro* Th2 polarization, Th2 cell differentiation can occur in the absence of either IL-4 or STAT6 when complemented by enforced STAT5 signaling (Zhu *et al.*, 2003) and previously published studies have shown that development of Th2 cells is unimpaired *in vivo* in the absence of either STAT6 or IL-4 during infection with *Nippostrongylus brasiliensis* (van Panhuys *et al.*, 2008). Additional pathways for the induction of Th2 cell differentiation involving Notch signaling have also been proposed and components of the Notch signaling pathway directly bind to sites within the promoter and enhancer regions of GATA3 and IL-4 respectively (Amsen *et al.*, 2009).

Similar to natural Treg, inducible or adaptive Treg differentiation in the periphery is often associated with expression of the transcription factor, Foxp3. However in contrast to natural Treg development in the thymus, it is sub-optimal TCR stimulation together with TGF-β and IL-2 that upregulates Foxp3 expression through the respective activation of NFAT, STAT5 and SMAD that bind to various regions of the Foxp3 promoter (Josefowicz and Rudensky, 2009). aTregs also exhibit increased expression of CD25 (IL-2Rα) and IL-2 is important in the selective expansion and survival of aTreg. Signals from the vitamin A metabolite retinoic acid (RA) can additionally promote aTreg development and enhance TGF-β-dependent induction of Foxp3 expression. Constant low level antigen stimulation from food products and commensal organsims coupled with the identification of $CD103^+$ DCs capable of producing TGF-β and RA (Coombes *et al.*,

2007; Sun *et al.*, 2007), favors the intestinal environment as a major site for peripheral aTreg induction and suggests a protective function for aTreg in suppressing inflammation at mucosal sites. However, the relative importance of natural versus aTreg *in vivo* remains to be determined.

Initiation of Th17 cell differentiation is dependent on TGFβ signaling in conjunction with IL-6-, IL-21, or IL-23-mediated STAT3 activation and expression of the transcription factor RORγt, while autocrine expression of IL-21 also serves as a positive feedback loop re-inforcing Th17 cell induction (Korn *et al.*, 2009). Recent work has placed the AP1 transcription factor BATF as a key player in Th17 cell induction (Schraml *et al.*, 2009) and it is likely that BATF cooperates with factors including RORγt and IRF4 (Brustle *et al.*, 2007) to enforce Th17 cell differentiation. The role of IL-23 in Th17 cell differentiation has remained controversial. Although *in vitro* studies have clearly shown that IL-23 is not required for the initiation of Th17 cell differentiation, consistent with the observation that naïve T cells do not express the IL-23R, the full and sustained differentiation and survival of Th17 cells appears to require IL-23, implicating IL-23 as a necessary feedback signal for the maintenance of Th17 cell responses *in vivo* (Korn *et al.*, 2009).

Since the discovery of the requirement for TGFβ in the differentiation of Th17 cells (Bettelli *et al.*, 2006; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006), it has become clear that there is a close relationship between the development of Th17 cells and aTreg cells, supported by the preferential induction of these two subsets within intestinal tissue and

the capacity of their signature transcription factors, Foxp3 and RORγt, to cross-regulate each other through direct protein-protein interactions (Ichiyama *et al.*, 2008; Zhou *et al.*, 2008). Additionally, cells co-expressing Foxp3 and RORγt have been identified in the murine small intestine that produce less IL-17 than RORγt single-expressors (Zhou *et al.*, 2008) and in humans, a $Foxp3^{+}IL\text{-}17^{+}$ peripheral regulatory T cell population has recently been identified (Voo *et al.*, 2009). These findings, taken together with a recent study demonstrating the ability of former Foxp3-expressing cells to adopt a pathogenic phenotype (Zhou *et al.*, 2009), support a more fluid, plastic model of helper cell differentiation than originally envisaged where lineage commitment is not a terminal step and Th cells may adopt an altered phenotype based on the interpretation of external cues. Further dissection of the factors, including signals from commensal organisms, that regulate the balance between Th17 and aTreg cell differentiation in the steady state may help identify individuals with a predisposition to inflammatory disease as well as provide new therapeutic targets for treatment.

1.2.3 Initiation of Th cell differentiation by DCs

Recognition of pathogens that elicit Th1 cell responses promotes DC activation, upregulation of MHC class II and co-stimulatory molecules and expression of IL-12 (Kapsenberg, 2003). As discussed above, APC-derived IL-12 can promote the initiation and maintenance of Th1 cell differentiation in multiple ways. First, IL-12 signaling and STAT4 activation induces the expression of IFN-γ in innate cells such as natural killer (NK) cells as well as T cells themselves and promotes the expression of T-bet. T-bet in turn induces expression of IL-12Rβ2, allowing for positive feedback of IL-12 signaling on activated $T\text{-}bet^{+}$ T cells. Differential expression of Notch Delta-like ligands by DCs has

also been proposed to contribute to Th1 cell development in part through inhibition of Th2 cell development (Sun *et al.*, 2008; Amsen *et al.*, 2004). The central role DCs play in the initiation of type 1 immunity and inflammation has been demonstrated in a number of depletion studies utilizing CD11c-diptheria toxin R (DTR) transgenic mice in which administration of DT results in selective apoptosis of $CD11c^+$ cells (Jung *et al.*, 2002). Depletion of $CD11c^+$ DCs results in the abrogation of Th1 cytokine-dependent immunity to intracellular pathogens as well as reduced inflammation in colitis models (Sapoznikov and Jung, 2008). Additionally, $CD11c^+$ DCs have been shown to be sufficient for the promotion of antigen-specific Th1 cell differentiation and Th1 cell-dependent immunity to *Leishmania major* using genetic restriction of MHC class II expression to $CD11c^+$ DCs (Lemos *et al.*, 2004; Lemos *et al.*, 2003). Both of these transgenic mouse models will be used in **Chapter 2** to interrogate the requirement and sufficiency of DCs in the initiation and maintenance of Th2 cell differentiation *in vivo*.

The recognition and response to helminths and protein allergens is less well understood than that of Th1 cell-inducing pathogens and as of yet, there are no known PRRs devoted to the promotion of Th2 cell differentiation leading some to suggest that these multi-cellular parasites may be too similar to their hosts to provide sufficiently unique, conserved ligands for pattern recognition to play a role in their detection (Medzhitov, 2009). Nevertheless, DCs pulsed *in vitro* with helminth antigens can promote Th2 cell differentiation and while DCs are not known to be major sources of IL-4, the expression of co-stimulatory molecules in including OX40L (Balic *et al.*, 2004; Ekkens *et al.*, 2003) and ICOS (Wilson *et al.*, 2006) as well as the differential expression of the Notch ligands *jagged1* and *jagged2* (Amsen *et al.*, 2004) have been implicated in Th2 cell

development. Recent studies suggest that other innate immune cells, including basophils, eosinophils, NKT cells and mast cells may complement DC activation by providing early sources of IL-4 to promote Th2 cell development *in vivo* (Min et al., 2006). The evidence for DC-mediated pattern recognition, alternative mechanisms of helminth detection by innate immune cells and how these pathways help drive type 2 immune responses *in vivo* will be discussed extensively below in section **1.3**.

There are multiple pathways by which DCs have been shown to participate in the induction of aTreg cells. In particular, as discussed above, $CD103^+$ intestinal DCs have been shown to promote peripheral Treg conversion through secretion of active TGF-β and RA (Coombes *et al.*, 2007; Sun *et al.*, 2007) and DCs conditioned by epithelial cell products such as TGF-β or TSLP are able to prime tolerogenic or non-inflammatory T cell responses (Rimoldi *et al.*, 2005). Several products from microbial pathogens and parasites have also been shown to influence DC function and promote concurrent development of immunoregulatory IL-10 producing regulatory T cells; this pathogen-induced pathway for Treg differentiation may be an evolutionarily conserved mechanism for prolonging both host and pathogen survivial (Belkaid and Oldenhove, 2008).

Depending on the context of their activation DCs can be a source of TGF-β and IL-6 to promote Th17 cell differentiation and are also innate cell sources of the amplifying Th17 cell cytokine IL-23. Consistent with a role for Th17 cells in defense against fungal pathogens, stimulation of DCs *in vitro* with zymosan, a fungal wall component that signals through TLR2 and Dectin-1, can induce IL-23 production and the development of

Th17 cells; another C-type lectin, Dectin-2 is associated with IL-17 production *in vivo* during fungal infection (Robinson *et al.*, 2009). Stimulation of DCs via TLRs is associated with induction of both IL-12 and IL-23, however the mechanisms that control preferential secretion of one or the other are still unclear. Interestingly, two forms of the same pathogen, the yeast versus hyphael form of *Candida albicans,* can differentially induce either IL-12 or IL-23 from DCs (Acosta-Rodriguez *et al.*, 2007). Thus the balance of molecular signals that control DC production of either IL-12 or IL-23 may be dictated by pathogen-specific signals and involve cross-talk between multiple PRR pathways including TLRs and C-type lectins.

In addition to the various instructive molecular signals described above, DCs may contribute to Th cell differentiation by virtue of prolonged interaction with antigen-specific T cells. A recent study has proposed a novel mechanism for the generation of T helper cell diversity through the evolutionarily conserved process of asymmetric cell division. In that study, T cells undergoing initial division following *in vivo* microbial stimulation exhibited unequal partitioning of multiple cell fate determinants and the resultant first two daughter cells phenotypically and functionally resembled either effector or memory lineages (Chang *et al.*, 2007). Differential allocation of molecular determinants including transcription factors required for Th cell differentation into Th1, Th2, Th17 or aTreg cell lineages via asymmetric cell division could thus also contribute to the generation of various T helper cell fates from a single antigen-specific responder (Reiner, 2007).

1.3 Innate recognition of helminth parasites and the initiation of type 2 immunity *in vivo*

The expulsion of gastrointestinal helminths is critically dependent upon the ability of the host to generate a polarized Th2 cell immune response and as such helminth infection has been a useful model for studying the development of type 2 immunity *in vivo* (Finkelman *et al.*, 2004; Maizels and Yazdanbakhsh, 2003). The Th2-associated cytokines IL-4, IL-5, IL-9, IL-13, IL-25 and IL-33 are known to play important roles in mediating the effector mechanisms that contribute to worm expulsion such as goblet cell hyperplasia and mucin production, eosinophilia, mastocytosis, recruitment of alternatively activated macrophages (AAMacs), increased epithelial cell turnover, and muscle hyper-contractility (Anthony *et al.*, 2007; Nair *et al.*, 2006). However, as discussed above, much less is known about the nature of the initiating events upstream of the adaptive immune response against helminth parasites. This section will review advances in our understanding of how innate immune cells recognize helminth-derived products and how this innate recognition is linked to the initiation of type 2 immune responses *in vivo*.

1.3.1 Helminth modulation of innate immunity through Toll-like receptors

DC recognition of microbes by TLR ligation typically results in their phenotypic maturation characterized by increased expression of co-stimulatory molecules CD40, CD80, and CD86, up-regulation of surface MHC class II, and the production of pro-inflammatory cytokines such as IL-6, IL-12, IL-23, and TNF (Kapsenberg, 2003). In contrast, helminth-matured DCs are notable for their relatively immature status; they often express low levels of co-stimulatory molecules and pro-inflammatory cytokines (MacDonald and Maizels, 2008) and in some cases are rendered refractory to

subsequent stimulation through TLR activation (Kane *et al.*, 2004). While less phenotypically mature, these DCs are capable of promoting robust antigen-specific $CD4^+$ T cell proliferation and Th2 cell differentiation and there is evidence that TLR signaling can play a role in the promotion of this "DC2" maturation program. Soluble egg antigen (SEA) preparations from the trematode *Schistosoma mansoni* are perhaps the best-characterized helminth-derived antigens that influence DC responses and contain a number of unique TLR ligands. For example, lacto-N-fucopentaose III (LNFPIII) is a milk sugar containing the $Lewis^x$ glycan that is found within SEA and can interact with TLR4 to selectively activate extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) signaling in DCs (Thomas *et al.*, 2003). LNFPIII exhibits Th2 adjuvanticity through both the recruitment of suppressor macrophages and the conditioning of DCs to promote $CD4^+$ T cell IL-4 production. Additionally, the schistosome-derived lysophosphatidylserine (lyso-PS) can interact with TLR2 to induce DCs that promote the differentiation of IL-4 and IL-10 producing T cells, similar to what some have observed following TLR2 ligation by Pam-3-cys (Dillon *et al.*, 2004a). Dependent upon the number of acyl chains, schistosome PS can differentially condition DC to promote either Th2 polarization or specifically induce IL-10 producing regulatory T cells (Treg) (van der Kleij *et al.*, 2002). It has been suggested that this induction of a Treg program by schistosome PS may be a means by which the parasite co-opts the normal host response to PS on the surface of apoptotic cells to suppress inflammation (van der Kleij *et al.*, 2002).

The filarial nematode ES product ES-62 is a phosphorylcholine-rich glycoprotein able to interact with TLR4 in a way distinct from that of the conventional TLR4 ligand, LPS. In

contrast to LPS, binding of ES-62 to TLR4 is independent of its Pro712 residue and ES-62 ligation of TLR4 on DCs and macrophages results in the inhibition, rather than promotion, of IL-12 secretion (Goodridge *et al.*, 2005). ES-62 is of particular interest in that it provides not only an innate signal to DCs and macrophages to limit pro-inflammatory cytokine production, but can also directly inhibit the effector functions of mast cells (Melendez *et al.*, 2007). Internalization of ES-62-TLR4 complexes by mast cells results in the sequestration and subsequent degradation of protein kinase C α (PKC-α), an important regulator of mast cell responses, leading to defective FcεRI-mediated mast cell degranulation and the selective inhibition of TNFα, IL-3, and IL-6, but not IL-5 and IL-13 release (Melendez *et al.*, 2007). Given the association of many intestinal helminth infections with the recruitment of large numbers of mast cells to the lamina propria, it is possible that the ability of nematode ES products to influence mast cell function could be a conserved evolutionary pathway to limit parasite-driven inflammation while selectively allowing expression of Th2 cytokines.

Despite the studies discussed above, a direct requirement for TLR signaling in generating anti-helminth type 2 immune responses remains controversial. While TLR ligation by helminth-derived antigens is recognized as a mechanism to limit the development of Th1 cytokine-mediated inflammation, it is still unclear whether signals induced by binding of helminth-derived antigens to TLR can directly promote Th2 cell differentiation. In fact, there is substantial evidence to suggest that the signaling pathways downstream of TLR are negatively correlated with the development of Th2 cytokine responses. For example, MyD88-deficient mice exhibit enhanced Th2 cytokine responses following exposure to *Trichuris* and are resistant to the development of

chronic infection (Helmby and Grencis, 2003). These data are consistent with the observation that MyD88$^{-/-}$ mice develop enhanced antigen-specific IL-13 responses following OVA immunization, proposed to be the result of defective Th1 cell differentiation and IFN-γ production (Schnare *et al.*, 2001) and that absence of the TLR/IL-1R–associated adaptor molecule, TNFR-associated factor 6 (TRAF6), results in a progressive Th2 cytokine-mediated inflammatory disease, (Chiffoleau *et al.*, 2003). It is important to note that these data do not exclude an important role for TLR signaling in the generation of anti-helminth immune responses independently of MyD88 and TRAF6. For example, other TLR-associated adapters including TIRAP, TIRP, TOLLIP and TRIF can influence multiple aspects of innate and adaptive immune responses although their role in recognition of helminth parasites awaits investigation. Similarly, the functions of intracellular PRRs including NOD proteins, NALPs, and NAIPs in innate responses to helminths are unknown. Given that some helminth parasites including *Trichuris* and *Trichinella spiralis* can inhabit a partially intracellular niche, these proteins may be biologically significant.

1.3.2 Helminth-associated glycans and C-type lectins in host-parasite interactions

The surfaces of helminths, as well as their excretory/secretory (ES) products, are rich in glycoproteins. Recognition of these carbohydrate domains is thought to be mediated by the calcium-dependent carbohydrate binding protein family of receptors, known as C-type lectins (C-TL), that are expressed by a number of innate cells including DCs, macrophages, and epithelial cells. The C-TL family consists of soluble and trans-membrane receptors that demonstrate unique specificity for carbohydrate residues via distinct clustering of carbohydrate recognition domains (CRD) and are able to distinguish

between sialylated and sulphated forms of the same carbohydrate (Cambi *et al.*, 2005). Trans-membrane members of the C-type lectin family include collectins, the mannose receptor family, selectins, and dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) and ligand engagement of these receptors is involved in processes ranging from cellular trafficking to cell signaling and pathogen recognition (Cambi *et al.*, 2005). Interaction between the peanut glycan allergen Ara h 1 and DC-SIGN has recently been shown to activate ERK MAPK signaling in DC and contribute to DC-mediated Th2 cell differentiation (Shreffler *et al.*, 2006). While glycans from schistosomes (Meyer *et al.*, 2005) and *Toxocara canis* (Schabussova *et al.*, 2007) have also been shown to interact with DC-SIGN, the functional consequences of these host-parasite interactions remains to be interrogated. The calcium-dependent galactose-binding proteins, intelectins, are expressed by paneth cells and goblet cells and have also been implicated in the recognition of gastrointestinal helminths. Although their functions in innate immunity remain largely unknown, intelectins are highly induced in the intestine following exposure to both *T. muris* and *T. spiralis* and have been proposed to interact with the surface of parasites to impede attachment to host surfaces (Artis, 2006; Pemberton *et al.*, 2004). The secretory glycoprotein, IPSE/alpha-1, found in SEA is an example of a helminth glycan that does not appear to associate with a classical C-TL for signaling but acts through IgE to induce the production of IL-4 from basophils in an antigen-independent way (Schramm *et al.*, 2007). This ability to elicit IL-4 production *in vivo* in the absence of antigen-specific IgE offers a potential mechanism for helminth induction of innate IL-4 that can contribute to Th2 cell differentiation.

1.3.3 Innate recognition of proteases

The natural life cycle of many helminths involves extensive migration through host tissues such as the skin, lung, and intestine. Traversing these tissues as larvae and establishing permanent residence in host tissues as adults is facilitated by the secretion of a number of parasite-derived proteases that allow the digestion of structural proteins like fibronectin, collagen, and laminin (McKerrow *et al.*, 2006; Falcone *et al.*, 2004). For a number of years it has been recognized that many protein allergens, such as house dust mite-derived *Der p 1*, *Fel d1* from domestic cats, and fungal allergens, also possess intrinsic protease activity (Falcone *et al.*, 2004). Similar to helminth parasites, these allergens most often gain access to host tissues at mucosal sites and in some individuals elicit potent Th2 cytokine responses with many of the hallmarks of helminth infection including eosinophilia, goblet cell hyperplasia, and elevated serum IgE levels. While the link between protease activity and type 2 inflammation is well documented, the mechanistic basis for these observations has been unclear.

Basophils, innate immune cells able to rapidly secrete IL-4 *in vitro* following stimulation with anti-IgE (Gessner *et al.*, 2005) as well as in response to allergens and helminth antigens (Phillips *et al.*, 2003), are poised at the interface between the innate sensing of antigens and the initiation of adaptive Th2 cytokine responses. Medzhitov and colleagues recently demonstrated that protease-mediated activation of basophils might be a shared link between allergens and helminth infection. Immunization with the cysteine protease allergen, papain, resulted in the transient recruitment of basophils to lymph nodes that peaked one day prior to the peak of IL-4 producing $CD4^+$ T cells (Sokol *et al.*, 2008). These papain-elicited basophils within the lymph node were shown to

express TSLP, an IL-7-like cytokine produced predominantly by epithelial cells and implicated in $CD4^+$ Th2 cell differentiation (Zhou *et al.*, 2005). *In vivo* depletion of TSLP or basophils correlated with impaired Th2 cell differentiation following papain immunization, suggesting a role for basophil-derived TSLP in papain-mediated Th2 cytokine responses. Interestingly, TSLP-TSLPR interactions are critical for immunity to *Trichuris*, although the dominant cellular source of TSLP was intestinal epithelial cells (Zaph *et al.*, 2007). Whether conserved mechanisms exist to promote expression of TSLP in multiple cell lineages is unclear at present. In **Chapter 3** of this thesis, we identify a novel role for TSLP in eliciting peripheral basophilia and investigate the role of basophils in initiation of the type 2 immune response to *Trichuris*.

Of note, treatment of basophils with papain *in vitro* also resulted in the production of IL-4, IL-6, and TNF but not histamine, reminiscent of the ability of helminth products, like ES-62, to selectively induce cytokine production while limiting release of pro-inflammatory mediators from innate effectors (Sokol *et al.*, 2008). While these data are consistent with a role for secreted proteases in the direct activation of basophils, the mechanism of host recognition remains unknown. Given their multiple roles in regulating inflammation, cellular trafficking, and epithelial barrier function members of the protease-activated receptor (PAR) family (Amadesi and Bunnett, 2004) are appealing potential molecular targets for helminth proteases. Recent work from Shea-Donohue and colleagues demonstrated that *Nippostrongylus brasiliensis* infection induced an IL-13-dependent increase in PAR-1 expression in the small intestine and that PAR-1 expression was associated with increased smooth muscle hypercontractility (Zhao *et al.*, 2005). Additionally, it was recently shown that proteases could signal through PAR family

members to induce TSLP expression from epithelial cells (Briot *et al.*, 2009; Kouzaki *et al.*, 2009).

Another potential mechanism by which allergen- or helminth-derived proteases may influence Th2 cytokine responses is via the degradation of IL-13Rα2. Prolonged exposure to house dust mite or mold allergens *in vitro* resulted in the cleavage and degradation of the soluble IL-13Rα2 (Daines *et al.*, 2007), a decoy receptor proposed to modulate IL-13R signaling (Chiaramonte *et al.*, 2003). Degradation of IL-13Rα2 resulted in enhanced IL-13R-dependent STAT6 activation and was dependent upon the protease activity of the allergens as protease inhibitor treatment abrogated the effect. Whether this mechanism is conserved between allergens and nematode ES protease products however is unknown.

1.3.4 Recruitment of innate effector cells by chitin

Chitin is one of the most abundant natural polymers on earth and is found in fungi, protozoa, insects, crustaceans, and parasitic nematodes. Recent studies have implicated chitin, as well as the enzymes involved in its degradation, in the etiology of airway inflammation and regulation of Th2 cytokine responses (Reese *et al.*, 2007; Zhu *et al.*, 2004). In nematodes, chitin provides support for structures such as the pharynx and is a key component of parasitic and free-living nematode egg shells; *H. polygyrus* eggs for example are composed of nearly 5% chitin by weight (Arnold *et al.*, 1993). Recently, it was demonstrated that the intranasal administration of chitin induced an accumulation of eosinophils and basophils, innate immune cells competent to produce IL-4, in the lung (Reese *et al.*, 2007), identifying a novel mechanism by which helminth-derived antigens may drive type 2 inflammation. Exposure to chitin also induced the

alternative activation of macrophages as indicated by the presence of arginase-expressing cells in the lung as early as 6 hours post-intranasal administration of chitin. The recruitment of innate immune cells was dependent upon both expression of the high affinity receptor for leukotriene B_4, BLT1, and upon the presence of macrophages as clodronate liposome depletion of macrophages prior to i.p. injection of chitin efficiently abrogated eosinophil recruitment to the peritoneum (Reese *et al.*, 2007), suggesting that macrophages may be an early cellular target for chitin recognition. The role of chitin in innate immune cell recruitment in the context of helminth infection models and the mechanism of chitin recognition are still unclear but represent exciting avenues of future research.

1.3.5 Activation of intestinal epithelial cells following helminth infection

IECs express a broad range of PRR including TLR, intracellular Nod proteins, and C-TL, as well as classical and non-classical MHC molecules involved in antigen processing and presentation (Shao *et al.*, 2005). The intimate association of intestinal dwelling nematodes with IECs, *Trichuris* for example lives partially embedded within IEC-derived syncytial tunnels (Tilney *et al.*, 2005), coupled with their expression of PRR and antigen-presenting machinery situate them as sentinels in recognition of helminth parasites in the intestine. Using IEC-specific knockout mice, our lab recently demonstrated that IEC-intrinsic NF-κB activation is critical in the development of anti-helminth Th2 cytokine responses (Zaph *et al.*, 2007). In that study, loss of NF-κB-dependent TSLP expression in IECs was correlated with exaggerated production of IL-12/23p40 and TNF by intestinal DCs, consistent with the development of an inappropriate Th1 response following *Trichuris* infection. TSLP can also act directly on mast cells (Allakhverdi *et al.*, 2007b) although the role of IEC-mast cell interactions in innate responses to helminth

parasites is unclear at present. In addition to TSLP, IECs are a major source of IL-25 and IL-33, two cytokines associated with the promotion of Th2 cytokine responses at mucosal sites. IL-25 appears to act directly on either $CD4^+$ T cells (Angkasekwinai *et al.*, 2007) or mast cells (Fallon *et al.*, 2006) to augment expression of Th2 cytokines and is critical for protective immunity following intestinal helminth infection (Owyang *et al.*, 2006). IL-33 can also directly promote Th2 cytokine production from $CD4^+$ T cells (Schmitz *et al.*, 2005) and activate mast cells (Allakhverdi *et al.*, 2007a). Recently IL-33 has been shown to accelerate worm expulsion when administered during *Trichuris* infection as well as mediate lymphocyte-independent pathological changes in the intestine (Humphreys *et al.*, 2008). A critical question is how IECs are activated following exposure to helminth parasites and what molecular pathways control expression of cytokines as diverse as TSLP, IL-25 and IL-33.

1.3.6 Helminth products trigger release of inflammatory mediators by granulocytes

As discussed above, granulocytes such as basophils, eosinophils, and mast cells are recruited early to sites of helminth infection and draining lymph nodes and are postulated to contribute to the generation and maintenance of $CD4^+$ Th2 cells through the rapid release of Th2-associated cytokines IL-4, IL-13, and, in the case of basophils, TSLP (Sokol *et al.*, 2008; Gessner *et al.*, 2005). In addition to cytokine production, recent studies have highlighted a novel group of endogenous inflammatory mediators derived from granulocytes, macrophages, and epithelial cells, called “alarmins” that can serve both as chemoattractants and provide maturation signals to DCs (Oppenheim and Yang, 2005). Among this group of mediators that includes defensins, cathelicidins, and high-mobility group box protein 1, the RNase A superfamily member eosinophil-derived

neurotoxin (EDN) has recently been shown to augment the capacity to DCs to promote Th2 cell differentiation in a TLR2-dependent manner (Yang *et al.*, 2008). The recruitment of eosinophils to mucosal sites following infection with several species of helminth parasites and during allergic responses suggests a potential role for EDN in initiating or maintaining Th2 cytokine responses. However, in most helminth infection models depletion of eosinophils via anti-IL-5 or anti-CCR3 treatment or genetic deletion has demonstrated that eosinophils are not critical for the generation of anti-helminth Th2 cytokine responses (Anthony *et al.*, 2007).

1.3.7 Macrophages and DCs in helminth infection

While *in vitro* stimulation with IFN-γ induces the generation of "classically activated" macrophages that produce IL-12 and inducible nitric oxide synthase (iNOS), exposure to Th2 cytokines is associated with the alternative activation of macrophages that express high levels of arginase (Gordon, 2003). As such, alternatively activated macrophages (AAMacs) are hallmarks of helminth-mediated inflammation and are abundant in schistosome egg-induced granulomas and in the lamina propria during intestinal helminth infection. These macrophages have a unique transcriptional profile characterized by expression of the mannose receptor, resistin-like molecule alpha (RELMα), and the chitinase-like molecule YM-1 (Nair *et al.*, 2003). While an essential role in mediating immunity during *H. polygyrus* challenge (Anthony *et al.*, 2006) and in limiting pathogenic inflammation in *S. mansoni* infection (Herbert *et al.*, 2004) have been attributed to AAMacs, their ability to influence the generation and/or function of Th2 cells is unknown and is an area of intense research. The regulation of macrophage antigen-presenting cell function by the Th2-associated cytokine IL-31 will be examined in **Chapter 4**.

DCs are a critical APC in the priming of naïve $CD4^+$ T cells and, as discussed above, can respond to helminth-derived products by promoting $CD4^+$ Th2 cell differentiation both *in vitro* and *in vivo*. However, the precise signals that DCs may provide T cells to promote Th2 cell differentiation are still unclear and the role of DCs in the initiation of Th2 cytokine-dependent immunity and inflammation will be investigated in **Chapter 2**. As discussed above, Amsen and colleagues have proposed that differential expression of Notch ligands Delta and Jagged by DCs may dictate Th1 and Th2 differentiation respectively (Amsen *et al.*, 2004). The defective Th2 polarization of antigen-specific T cells following co-culture with ovalbumin-pulsed *jagged2*$^{-/-}$ DC support these observations, although the requirement for *jagged2* in Th2 cell differentiation was not replicated *in vivo* (Worsley *et al.*, 2008). Notwithstanding this, recent studies have demonstrated that T cell-intrinsic Notch activation is critical for optimal Th2 cell differentiation and cytokine production *in vivo* following *Trichuris* infection (Tu *et al.*, 2005) likely via direct activation of the Th2 transcription factor GATA3 (Amsen *et al.*, 2007; Fang *et al.*, 2007). In addition to the expression of Notch ligands, DC may directly influence Th2 cell differentiation through expression of co-stimulatory molecules, OX40L and CD40, which have been implicated in the generation of optimal Th2 cell responses (Jenkins *et al.*, 2007; MacDonald *et al.*, 2002). Thus, the integration of signals from helminth-activated innate cells such as IECs, granulocytes, macrophages and DCs are likely to be involved in the initiation of optimal Th2 cell differentiation (**Fig. 4**).

1.4 *Trichuris muris*: A model of type 2 immunity and inflammation in the GI tract

As discussed above, helminth infection provides a natural *in vivo* model with which to study the development of type 2 immunity and inflammation. Infection with the enteric nematode *Trichuris muris* is a model that will be used throughout this thesis; therefore this section will introduce the biology of *Trichuris* infection and the factors that govern resistance and susceptibility to infection.

1.4.1 **Lifecycle and physiological niche of *Trichuris muris***

Trichuris utilizes a fecal/oral route of transmission and has a direct lifecycle whereby eggs are ingested by the host, hatch in the cecum and undergo three sequential molts before reaching sexual maturity, after which adult worms release eggs into the lumen of the gut to continue the cycle (Cliffe and Grencis, 2004); in humans, the egg output of an adult female is estimated at between 3000-5000 per day (Bethony *et al.*, 2006). These eggs are extremely stable in the environment and have even been recovered from mummies. Once hatched, *Trichuris* resides in the lumen of the cecum and proximal colon of mice where it remains in close association with host epithelial cells. The anterior end of *Trichuris* is embedded within IECs in what have been termed “syncytial epithelial tunnels” (**Fig. 5**) while the posterior end of adult worms protrudes freely in the lumen to allow for mating and the deposition of eggs. Recent work from our lab has confirmed that *Trichuris* is in direct contact with the cytoplasm of mucosal epithelial cells but travels through the luminal surface of the epithelium and does not penetrate the basement membrane, leaving the lamina propria intact (Tilney *et al.*, 2005). Chronic infection with *Trichuris* and other soil transmitted helminths results in the sequestration of nutrients from the host and in humans is associated with malnutrition, stunted growth

and impairment of cognitive development in children (Bethony *et al.*, 2006). As of yet, there are no successful vaccines for the preventative treatment of soil-transmitted helminths and continued drug treatment for de-worming represents not only a significant economic burden but in some cases may no longer be efficacious due to the emergence of drug-resistance within populations (Wolstenholme *et al.*, 2004). Thus, there is a growing need for the identification of novel vaccine and therapeutic targets in defense against this class of pathogens.

1.4.2 Immunity to *Trichuris*

Experimental infection with the murine intestinal nematode *Trichuris* offers a tractable model of $CD4^+$ T cell-dependent immunity and inflammation in the gut whereby genetically susceptible and resistant strains of mice develop polarized Th1 and Th2 cytokine responses respectively. Depletion experiments, in combination with adoptive transfers of lymphocytes into SCID mice, have demonstrated that $CD4^+$ T cells are both necessary and sufficient for protection during *Trichuris* infection and *in vivo* manipulation of cytokines by gene deletion or antibody depletion has emphasized the importance of the Th2 cytokines IL-1, IL-4, IL-13, and IL-25 in resistance to infection (Cliffe and Grencis, 2004). The effector mechanisms by which Th2 cytokines mediate expulsion of *Trichuris* are incompletely understood but include the “weep and sweep” response, associated with increased mucin secretion and permeability of the intestine and an IL-13-dependent increase in epithelial cell turnover or “epithelial escalator” that acts to displace *Trichuris* from its habitat (Cliffe *et al.*, 2005). As discussed in section **1.3**, while the effector responses required for the expulsion of gastrointestinal nematodes are beginning to be understood, the cell types that are responsible for the recognition of

these pathogens and the signals that direct a protective Th2 cytokine response are unclear.

Over a billion people worldwide are estimated to be infected with *Trichuris* species and available human data correlates with many of the observations made in experimental murine models of Trichiuriasis. For example, there is evidence that as in mice there are individuals with a potential genetic pre-disposition to susceptibility or resistance to infection. In a study following individuals who were naturally infected with *Trichuris* and treated, re-infection rates and worm burdens remained steady over time, with initial infection burdens highly positively correlated with re-infection burdens (Bundy *et al.*, 1988). Additionally, weak peripheral blood Th2 cytokine responses were demonstrated to be predictive of susceptibility to infection and re-infection of humans with *Trichuris* (Jackson *et al.*, 2004), supporting murine studies demonstrating the requirement for a highly polarized Th2 cytokine response in immunity and highlighting the potential impact of murine studies on human disease.

1.5 Regulation of immunity and inflammation by the gp130 group of cytokines and cytokine receptors

While determining the factors that initiate productive immune responses to pathogens is vital for the identification of putative new vaccine targets and adjuvants to fight infectious disease, it is equally important to decipher the pathways involved in the negative regulation of inflammation. Unchecked inflammatory responses, whether elicited by infection, allergen exposure or autoimmune reactions, are often associated with host tissue damage and can drive multiple pathologies including dermatitis, inflammatory bowel disease, multiple sclerosis, asthma and arthritis. Therefore, an intense area of

research is identifying factors with therapeutic potential that can negatively regulate immune-mediated inflammation. Cytokines are soluble factors secreted by cells that can act in an autocrine or paracrine manner to influence cellular behavior and, as one of the largest groups of extracellular regulatory molecules (Boulanger and Garcia, 2004), are appealing candidates for manipulation in inflammatory diseases. The largest family of cytokines and cytokine receptors is the type 1 family and within this family, members within the gp130 group have proven critical mediators of both pro- and anti-inflammatory responses in multiple disease settings. The role of one of the more recently identified members of this family of cytokines, IL-31, in the regulation of type 2 immune responses at mucosal sites will be investigated in **Chapter 4**. Here, I will introduce the gp130 cytokine/cytokine receptor group and focus on the roles of the prototypic member, IL-6, as well as the closely related IL-31 in regulating immunity and inflammation.

1.5.1 The gp130 group of cytokine receptors

Clustered together on the basis of sequence, structure and functional homology, the gp130 group of cytokine receptors includes gp130, IL-6Rα, IL-11Rα, IL-12Rβ1, IL-12Rβ2, CNTFRα, IL-23Rα, WSX-1, OSMR, G-CSFR, LIFR and GLMR (or IL-31Rα) (Boulay *et al.*, 2003) and is the largest subgroup within the type 1 cytokine receptor family. Most cytokine receptors are dimeric complexes composed of a “private” ligand-specific receptor and a “public” shared signal-transducing receptor that dictates some of the conserved functional properties within members of a cytokine family (Kishimoto *et al.*, 1994). As the name would suggest, for the gp130 family this “public” receptor is the gp130 receptor or closely related gp130-like receptors including IL-12Rβ2, IL-23Rα and IL-31Rα. While many of the gp130 family members have overlapping or redundant

functions, they also exhibit notably divergent functions attributed to their unique cytokine receptor chain expression, cell type specific expression patterns and promiscuous ligand-receptor interactions.

A common signaling pathway activated downstream of cytokine receptor ligation is the Janus tyrosine kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway. Ligand engagement of members of the gp130 receptor family is associated with the phosphorylation and activation of a broad range of STAT proteins, including STAT1, STAT3, STAT4 and STAT5, and the differential activation of these proteins is thought to contribute to the pleitropic functions of gp130 receptors. STAT1 and STAT4 for example, the predominant STATs activated by IFN-γ and IL-12 respectively, are often associated with pro-inflammatory responses and promotion of Th1 cell differentiation while STAT3 signaling is linked to both the anti-inflammatory functions of regulatory cytokines like IL-10 and the pro-inflammatory functions of IL-6; STAT5 phosphorylation, a common response to activation of members of the common gamma chain (γC) cytokine receptor family, can promote cell growth and survival. The above are over-simplified examples of responses associated with STAT phosphorylation and the magnitude and duration of intracellular signaling pathways are principle modulators of the functional outcome of receptor ligation. Suppressor of cytokine signaling (SOCS) proteins are an example of specific negative regulators of STAT activation that are concomitantly induced following receptor activation, thus representing a typical negative feedback signal to control cytokine signaling. SOCS proteins exert their regulatory activity through multiple mechanisms, including direct inhibition of JAK signaling via their kinase inhibitory region (KIR) and ubiquitin-mediated proteosomal

degradation of the signaling complex. The importance of SOCS protein expression in regulating cytokine responses has further been demonstrated by genetic deletion of specific SOCS proteins and transgenic expression of receptors with mutations within SOCS binding sites (Yoshimura *et al.*, 2007).

1.5.2 Functional biology of IL-6

IL-6 is the prototypic member of the gp130 group cytokines and has a four helix bundle structure that is conserved amongst α helical cytokines within the gp130 group, of which IL-31 is the most structurally similar to IL-6. When both subunits of the IL-6R, IL-6Rα and gp130, are expressed on the target cell surface, IL-6 binding induces gp130 homodimerization and signaling through a hexameric IL-6/IL-6Rα/gp130 complex (Boulanger *et al.*, 2003). There is also a soluble form of IL-6Rα that can be generated by proteolytic cleavage from the cell surface or de novo synthesis from differential mRNA splicing that can bind to both IL-6 and ubiquitously expressed membrane-bound gp130 to promote trans-signaling in cells that do not intrinsically express IL-6Rα. This trans-signaling allows the propogation of IL-6-dependent responses within multiple cell types during acute and chronic inflammation. For example, the shedding of soluble IL-6Rα (sIL-6R) by activated neutrophils during acute inflammation allows endothelial cell production of chemokines and integrins that promote leukocyte recruitment and this trans-signaling is essential for the eventual resolution of inflammation in human and mouse models of peritonitis (Boulanger *et al.*, 2003). However, in the context of models of colitis, IL-6 trans-signaling inhibits the apoptosis of lamina propria T cells thereby promoting chronic inflammation and this inflammation can be relieved by treatment with a gp130-Fc protein that blocks sIL-6R from associating with cell-bound gp130 (Atreya *et*

al., 2000). Many factors can contribute to the diverse functional outcomes of IL-6 signaling and, as discussed above, differential expression of SOCS proteins may in part explain some of these effects. For example, a series of papers analyzing IL-6 responses in mice with a lineage-specfic deletion of SOCS3 in macrophages and neutrophils demonstrated that sustained IL-6/STAT3 signaling in the absence of SOCS3 resulted in anti-inflammatory macrophage responses similar to those following IL-10 treatment (Lang *et al.*, 2003; Yasukawa *et al.*, 2003). IL-6 signaling is thus associated with a variety of both pro- and anti-inflammatory activities depending on the context of stimulation, receptor expression levels, the cell type analyzed, and the activity of SOCS proteins.

1.5.3 IL-31-IL-31 receptor signaling and function

The gp130-like monocyte receptor (GLMR), now known as IL-31Rα, is one of the most recently identified members of the gp130 group and bears the closest homology to gp130. In fact, gp130 and IL-31Rα are located on the same chromosome and IL-31Rα has been proposed to be the result of gene duplication of gp130. The IL-31Rα pairs with oncostatin M receptor (OSMR) to form the signaling receptor for IL-31 (**Fig. 6**). Expression of IL-31Rα has been identified on monocyte/macrophage populations, epithelial cells and T cells and its ligand, IL-31, is expressed by activated T cells, preferentially of the Th2 cell lineage, and has recently been reported to be expressed by some granulocyte populations associated with type 2 inflammation such as basophils and mast cells (Ishii *et al.*, 2009; Sokol *et al.*, 2008). The major pathways activated downstream of IL-31R signaling are STAT1, STAT3 and STAT5 as well as MAP kinase signaling and *in vitro* activation via IL-31R signaling can elicit production of cytokines

and chemokines from epithelial cells as well as influence cell proliferative responses. For example, IL-31 stimulation of lung epithelial cells results in activation of STAT3, ERK, JNK and Akt pathways and is associated with the inhibition of proliferation via alteration in expression of cell cycle proteins (Chattopadhyay *et al.*, 2007). The signaling responsible for this alteration of cell cycle proteins was linked directly to IL-31Rα chain signaling and could be disrupted by mutation of a single STAT3-recruitment site within the intracellular signaling domain. IL-31 treatment of human bronchial epithelial cells can also increase production of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein MCP-1/CCL2, supporting a potential role for IL-31 in recruitement of immune cells to the lung (Ip *et al.*, 2007). While no known function has yet been assigned to it, there is a putative soluble IL-31Rα isoform (Dillon *et al.*, 2004b) and, given the close relationship between IL-31/IL-31Rα and IL-6/gp130, it remains a possibility that trans signaling through this receptor may be an important factor in determing the functional biology of IL-31 signaling *in vivo*. Alternatively, this soluble receptor may function similarly to IL-13Rα2 in negatively regulating IL-31 function. In support of this, administration of a soluble IL-31Rα-Fc protein partially blocked IL-31 signaling *in vitro* (Dillon *et al.*, 2004b).

The first evidence supporting a role for IL-31 in inflammatory disease *in vivo* came from an overexpression study where IL-31 was expressed either under a ubiquitous or T cell-specific promoter. In both cases, IL-31 overexpression resulted in the development of inflammation in the skin resembling atopic dermatitis with thickening of the epidermis and dermis, granulocyte recruitment, alopecia and pruritis (Dillon *et al.*, 2004b). Subsequent studies have highlighted a strong correlation between serum and tissue

levels of IL-31 and human dermatitis patients compared to normal controls and, in animal studies, it appears that IL-31 may be responsible for prurutis associated with dermatitis as blockade of IL-31 significantly reduces scratching behaviour in affected mice (Grimstad *et al.*, 2009). The development of skin inflammation resembling atopic dermatitis in mice overexpressing IL-31 coupled with the association of IL-31 with human dermatitis patients suggested that IL-31 may be a novel IL-6 family cytokine associated with the promotion of type 2 inflammation. However, no evaluation of cytokine expression was carried out in IL-31 transgenic mice and there was no detectable elevation in IgE observed during disease. The role of endogenous IL-31R signaling on the regulation of type 2 inflammation will be examined in **Chapter 4** via the generation of IL-31Rα deficient mice and the use of two models of Th2 cytokine-dependent inflammation in the intestine and the lung.

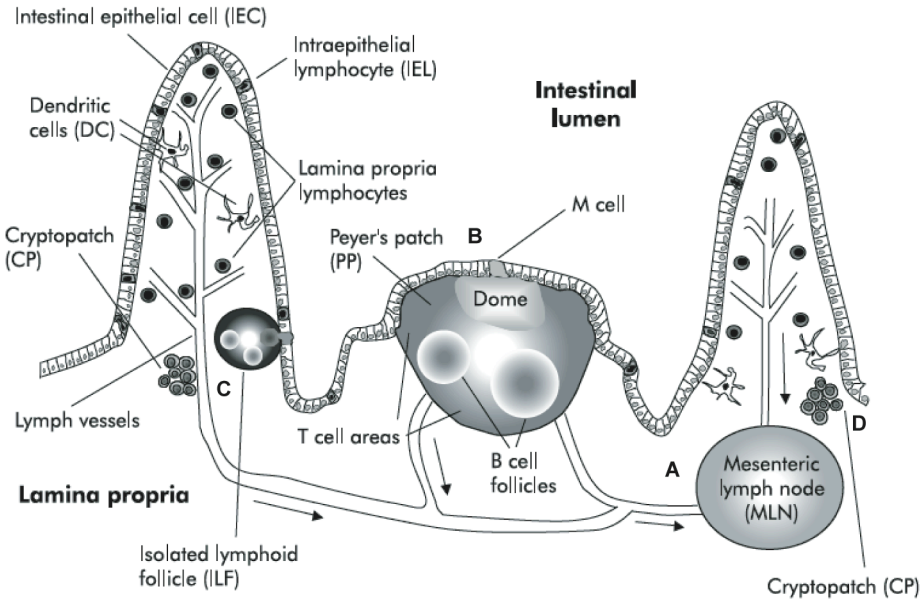

Figure 1. Anatomy of the intestine. Illustration of the architecture of the small intestine highlighting the major lymphoid structures including the mesenteric lymph nodes (A), Peyer's patches (B), isolated lymphoid follicles (C) and cryptopatches (D).

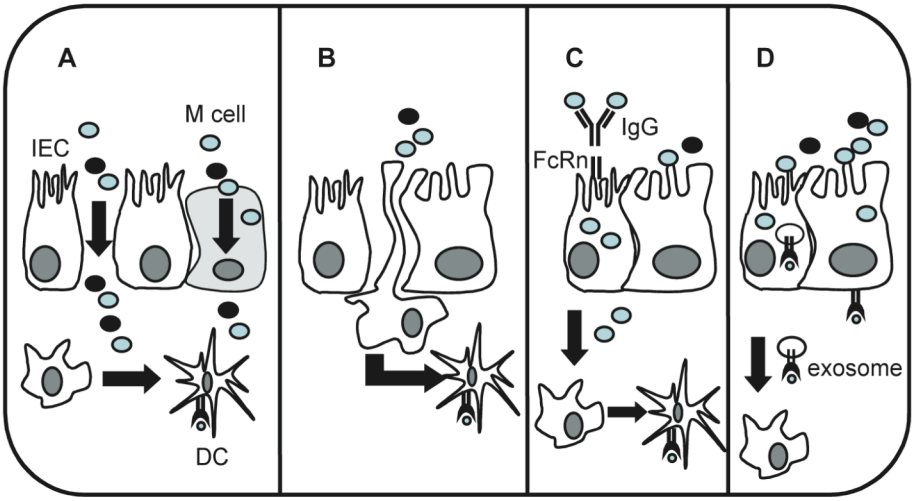

Figure 2. Antigen sampling in the intestine. Proposed mechansims for sampling antigens from the lumen of the intestine. (A) Antigen may gain access to the lamina propria (Lp) via a physical break in the intestinal barrier or by active transport by M cells located within the follicle associated epithelium and taken up by Lp dendritic cells. (B) Dendritic cell subsets within the small intestine Lp can extend their dendrites between tight cells junctions and directly sample antigens in the lumen. (C) In humans, neonatal Fc receptor expression on IEC is thought to facilitate uptake of antigen bound by IgG. (D) IEC can express MHC class II and may present antigens to T cells directly or indirectly.

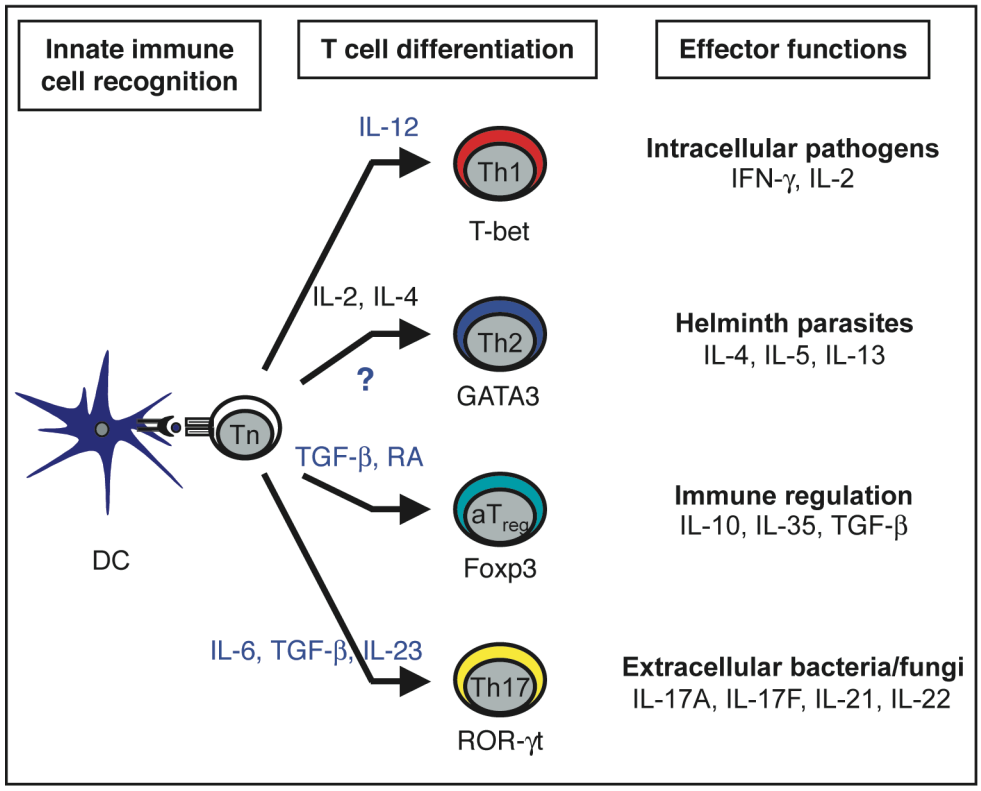

Figure 3. Helper T cell fates. $CD4^+$ T helper cell fates defined by mechanisms by which they develop from naïve T cells, unique cytokine and transcription factor profiles and their functions. The cytokines that are produced by DCs and are known to promote the initiation and/or maintenance of Th cell differentiation are highlighted in blue font.

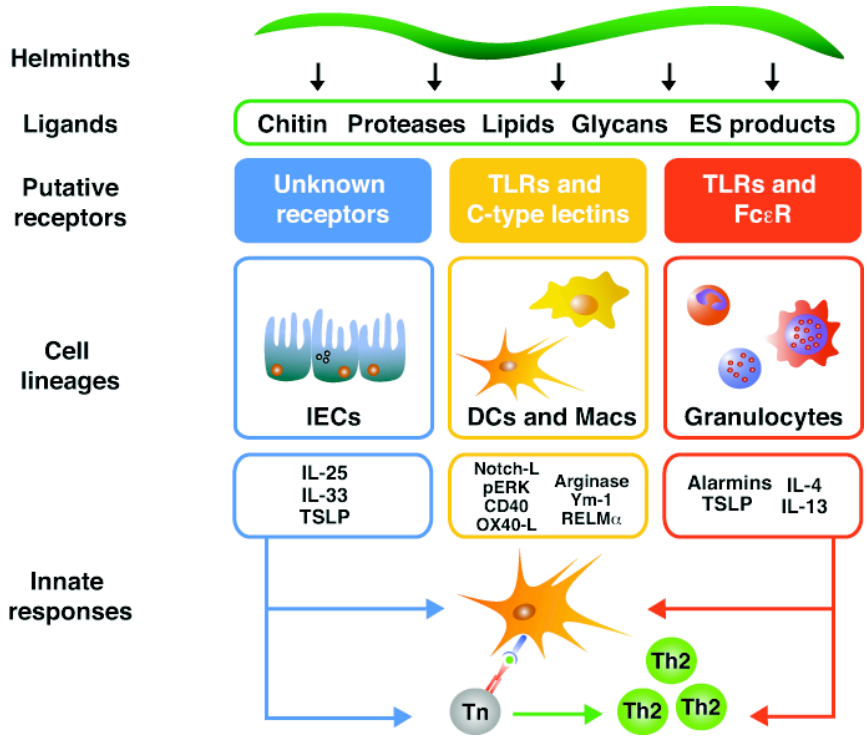

Figure 4. Coordinated initiation of type 2 immunity during helminth infection. Recognition of some helminth-derived products by innate cells can be mediated by germline encoded pattern recognition receptors such as TLR and lectins in addition to other defined and undefined biochemical recognition mechanisms. Innate cell responses include secretion of effector molecules such as IL-4, IL-13, IL-25, IL-33, TSLP, and "alarmins" that contribute to $CD4^+$ Th2 differentiation through influencing antigen presenting cell function and/or directly acting on $CD4^+$ T cells. DC conditioned with some helminth products can promote $CD4^+$ Th2 differentiation through changes mechanisms including expression of OX40L and CD40 and differential expression of Notch ligands.

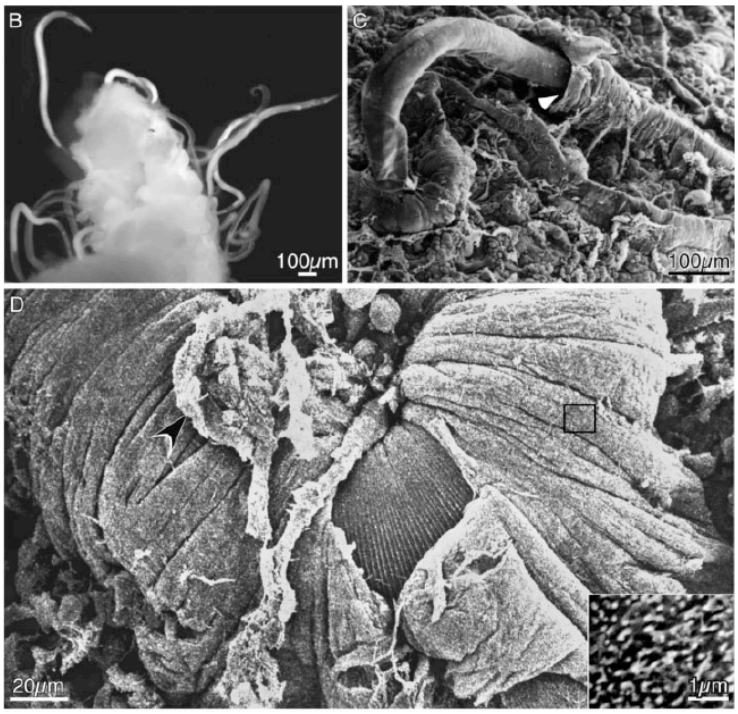

Figure 5. *Trichuris muris* inhabits a partially intracellular niche within the large intestine. Light microscopy and scanning electron microscopy images of *Trichuris* within the cecum of a chronically infected mouse. (Adapted from Tilney *et al* 2005)

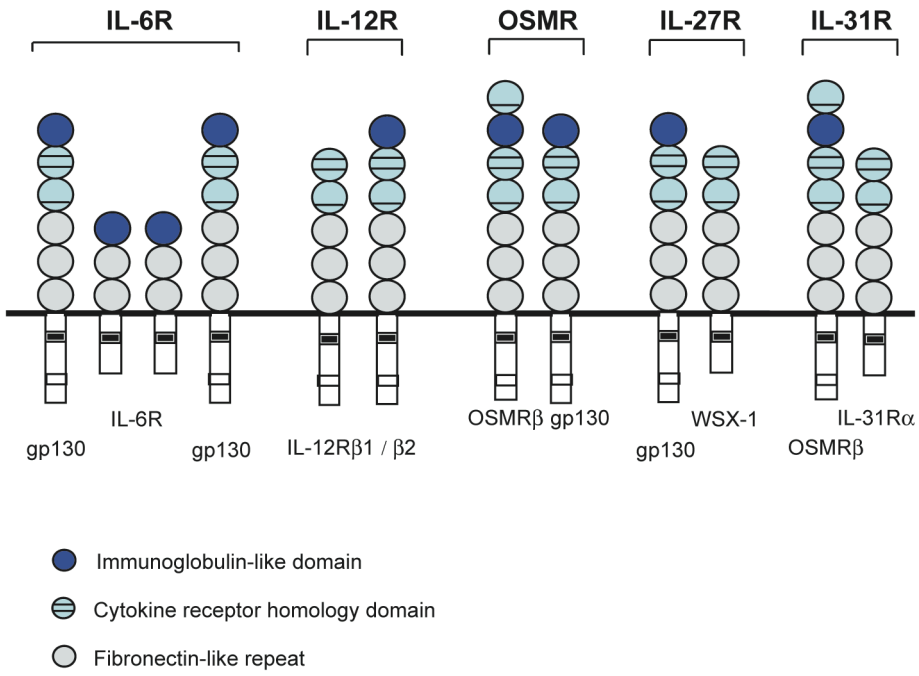

Figure 6. Selected members of the gp130 cytokine receptor family. Members of the gp130 family of cytokine receptors are grouped together on the basis of sequence and structural homology.

Chapter 2

$CD11c^+$ dendritic cells are insufficient for the development of Th2 cytokine-dependent immunity to *Trichuris*

2.1 Abstract

Protective immunity to helminth parasites is dependent upon the generation of $CD4^+$ T helper (Th) cell 2 cytokine responses characterized by production of IL-4, IL-5, and IL-13. While significant advances have been made in understanding the effector mechanisms downstream of this module of adaptive immunity, definition of the innate responses that promote $CD4^+$ Th2 cell responses and type 2 inflammation at mucosal surfaces remains fragmented. In this chapter, we demonstrate that dendritic cell (DC)-restricted expression of MHC class II, while sufficient for the priming and expansion of $CD4^+$ T cells, is insufficient for the development of Th2 cell-dependent immunity following infection with the intestinal helminth *Trichuris muris*. Transient depletion of DCs revealed that $CD11c^+$ DC contributed to the optimal expansion of $CD4^+$ T cells. However, neither DCs, macrophages nor B cells were required for Th2 cytokine-dependent immunity to *Trichuris* suggesting that additional non-professional APC-$CD4^+$ T cell interactions were required for the generation and/or maintenance of Th2 cells.

2.2 Introduction

Since the demonstration of $CD4^+$ T helper (Th) cell specification (Mosmann *et al.*, 1986), significant advances have been made in delineating the regulatory mechanisms that promote distinct modules of $CD4^+$ T cell-dependent immunity and inflammation (Reiner, 2007). In the case of Th2 cells, their differentiation is dependent on IL-4R, STAT6, and

GATA3 and their signature cytokine profile is characterized by expression of IL-4, IL-5, IL-9, and IL-13 (Mowen and Glimcher, 2004). The hallmarks of Th2 cytokine-dependent inflammation at barrier surfaces such as the skin, airway, and intestine include the recruitment of $CD4^+$ Th2 cells, eosinophils, mast cells, and basophils, coupled with goblet cell hyperplasia, mucus production, and increased smooth muscle contractility (Anthony *et al.*, 2007). These type 2 inflammatory responses are required for immunity and tissue repair following exposure to helminth parasites. However, Th2 cytokine responses can also promote pathological changes observed in the context of asthma and allergic diseases (Cohn *et al.*, 2004).

While the sequelae of type 2 immunity and inflammation in peripheral tissues is well characterized, the innate responses that promote Th2 cell development, including the nature of the antigen-presenting cell (APC) involved, the host-microbial receptor-ligand interactions, and the APC-derived factors required to initiate and sustain Th2 cell differentiation remain less well defined (Perrigoue *et al.*, 2008). DCs are the only APC thought to prime naïve T cells and the current paradigm suggests that recognition of conserved pathogen-associated molecular patterns via distinct pattern recognition receptors on DCs promotes appropriate pathogen-specific $CD4^+$ Th cell responses (Medzhitov, 2007). Activation of DCs can result in increased surface expression of MHC class II and co-stimulatory molecules such as CD40, CD80, and CD86 as well as expression of factors that can shape the nature of the developing adaptive immune response (Kapsenberg, 2003). For example, recognition of viral-, bacterial- or protozoan-derived microbial products via Toll-like receptors (TLR) can activate plasmacytoid and conventional DCs and promote production of type 1 interferons and IL-

12 that elicit adaptive cytotoxic $CD8^+$ T cell and $CD4^+$ Th1 cell responses. In addition, following stimulation with fungi and some extracellular bacteria, DC production of IL-6 and IL-23, in conjunction with TGFβ, promote the generation and maintenance of $CD4^+$ Th17 cells (Chen and O'Shea, 2008) associated with protective immunity (Matsuzaki and Umemura, 2007). In contrast, the critical DC-derived signals responsible for driving Th2 cell responses *in vivo* remain undefined (MacDonald and Maizels, 2008). *In vitro* studies indicate that the requirements for DC-mediated Th2 cell differentiation include differential expression of the Notch ligand *jagged* (Amsen *et al.*, 2004) and up-regulation of the co-stimulatory molecules CD40 (MacDonald *et al.*, 2002) and OX40L (Ekkens *et al.*, 2003). However whether these pathways are sufficient for DC to promote $CD4^+$ Th2 cell differentiation *in vivo* is unclear. Collectively, the inability of DC to express IL-4 and the lack of defined mechanisms through which DC promote Th2 cell differentiation have provoked a reassessment of the relative contribution of DC in promoting Th2 cytokine responses *in vivo*.

In this chapter, we demonstrate that DC-restricted expression of MHC class II is insufficient for the generation of protective $CD4^+$ Th2 cytokine-dependent immunity to the gastrointestinal helminth *Trichuris*. Additionally, transient depletion of dendritic cells using CD11c-DTR mice suggested that $CD11c^+$ cells were involved in the efficient expansion of $CD4^+$ T cells but may not be required for protective type 2 immune responses. In addition, liposome-clodronate depletion of macrophages and genetic deletion of B cells did not alter the Th2 cytokine response or worm expulsion following *Trichuris* infection. Collectively, these data suggest that additional non-professional APCs may be required for the development of type 2 immunity and inflammation *in vivo*.

2.3 Methods

2.3.1 Mice and parasites

6-8 week-old C57BL/6 mice and timed pregnant female B6.SJL mice were ordered from The Jackson Laboratories (Bar Harbor, ME). MHC II^{CD11c} (generated as previously described(Lemos *et al.*, 2003)), MHC $II^{-/-}$, μMT and CD11c-DTR mice were bred and housed in specific pathogen-free conditions at the University of Pennsylvania. Littermate control mice were sham-grafted and MHC II^{CD11c} mice were given subcutaneous thymic grafts from neonatal (0-2 days) B6.SJL mice at 4-6 weeks of age and allowed 8 weeks to reconstitute $CD4^+$ T cells before experimental use. Bone marrow chimeras were generated by i.v. injection of 5 million bone marrow cells from WT or CD11c-DTR mice into irradiated (2 x 500rads) WT recipients. Recipient mice were on antibiotics for two weeks and allowed 8 weeks for reconstitution of hematopoietic cells. All experiments were performed following the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. *Trichuris muris* was maintained in genetically susceptible mouse strains and antigen and eggs harvested as previously described (Zaph *et al.*, 2006). Briefly, infected susceptible mice were sacrificed at day 34 post-infection and adult worms isolated from the ceca and placed in RPMI with 5X penicillin/streptomycin. Media collected at 4 hours was dialyzed 3 times against PBS and filtered for use as antigen. Eggs secreted by adult worms were collected from media after both 4 and 24 hours of culture, washed with water, filtered over a 70μm cell strainer and allowed to embryonate in the dark for six weeks. Mice

were infected by oral gavage with 200-300 embryonated (as determined by microscopy) *Trichuris* eggs.

2.3.2 Polyclonal T cell stimulation

mLN were harvested and single-cell suspensions prepared in complete media (DMEM supplemented with 10% heat-inactivated FBS, 2mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25mM HEPES, and 5 x 10^{-5} M beta-mercaptoethanol). mLN cells were seeded in 48-well plates at 2.5x10^{6} per well and incubated with either media or 1μg/ml soluble anti-CD3/anti-CD28 (eBioscience) for 48 hours. Cell-free supernatants were harvested and cytokine production determined by standard sandwich ELISA. All antibody pairs were purchased from eBioscience: clones AN-18, R4-6A2 (IFN-γ), 11B11, BVD6-24G2, (IL-4), TRFK5, TRFK4 (IL-5) and eBio13A, eBio 1316H (IL-13).

2.3.3 Western blot

Fecal protein isolation was performed as previously described (Artis *et al.*, 2004b). Breifly, fecal pellets were homogenized in 500ul of U9 buffer (9M urea, 2% CHAPS, 50mM Tris-HCl pH 9.0) and incubated at room temperature 30 minutes. An additional 500ul of U9 buffer (diluted 1:10 in 50mM Tris-HCl) was then added and debris spun at 14k RPM for 10 minutes. Supernatant was analyzed by BCA assay for protein content and 30 μg of protein was loaded per sample for analysis by SDS-PAGE and immunoblotted for RELMβ using a polyclonal rabbit α-murine RELMβ antibody (Peprotech, Rocky Hill, NJ).

2.3.4 Real time quantitative PCR

RNA was isolated from intestinal tissues of mice using a TRizol extraction (Invitrogen) and from mLN cells using RNEasy Spin Columns (Qiagen). Tissues were disrupted in a

tissue homogenizer (TissueLyzer, Qiagen) and cDNA synthesized using Superscript Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was carried out on cDNA samples using commercial primer sets (Qiagen) and SYBR Green chemistry. All reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Samples are normalized to naïve controls unless otherwise stated.

2.3.5 Histology and immunofluorescence

Cecal tips were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. 5 μm sections were cut and stained for hematoxylin and eosin (H&E) or Alcian blue/periodic acid-Schiff. Unstained 5μm sections prepared on immunoslides were stained for Gob5 by immunofluoresence (Zaph *et al.*, 2007). Samples were de-paraffinized by consecutive methanol and ethanol rinses, boiled in citric acid buffer for 10 minutes and stained with an antibody against Gob5 at 4 degrees overnight followed by staining with a Cy2-conjugated anti-goat antibody (Jackson Immunoresearch) for 2 hours at room temperature. Samples were then rinsed with PBS and water, stained with DAPI for 60s and cover-slipped for analysis by microscopy.

2.3.6 Antibody treatment

Neutralizing monoclonal antibody against IFNγ (XMG-6) was purified from ascites by ammonium sulfate precipitation and dialyzed against PBS. Mice were given 1 mg antibody i.p. every 3-5 days during the course of infection starting at day 0.

2.3.7 Macrophage and dendritic cell depletion

PBS- or Clodronate-loaded liposomes were prepared as previously described(Van Rooijen, 1989). 150 ul of liposomes were injected i.v. every 2 days during the course of

infection. Clodronate was a gift from Roche Diagnostics GmBH (Mannheim, Germany). DCs were depleted in the CD11c-DTR mice by injection of 100ng diphtheria toxin (Sigma) i.p. per mouse (approximately 4ng/g body weight) every 72 hours during the course of infection.

2.4 Results

2.4.1 CD11c-restricted MHC class II expression is insufficient to generate $CD4^+$ Th2 cell-dependent immunity following intestinal helminth infection

To test whether antigen presentation by $CD11c^+$ DC was sufficient to promote $CD4^+$ Th2 cell-dependent immunity *in vivo*, mice in which MHC class II expression is restricted to $CD11c^+$ cells (MHC II^{CD11c} mice) were infected with the intestinal helminth parasite *Trichuris muris*. Expulsion of *Trichuris* and protective immunity is dependent on $CD4^+$ Th2 cells while parasite-specific IFN-γ production promotes chronic infection (Cliffe and Grencis, 2004; Else and Finkelman, 1998; Else *et al.*, 1994). *Trichuris* infection therefore provides a well-characterized *in vivo* model of Th2 cytokine-dependent immunity that offers a functional read-out of the magnitude of the host Th2 cytokine response. MHC II^{CD11c} mice are I-$Ab^{-/-}$ mice that contain a transgene with I-Ab expression under the control of the CD11c promoter, thereby restoring MHC class II expression only to $CD11c^+$ DCs (Lemos *et al.*, 2003) (**Fig. 7a**). Since MHC II^{CD11c} mice lack MHC class II expression on the thymic epithelium (Lemos *et al.*, 2003) and are therefore unable to positively select $CD4^+$ T cells in the thymus, mice were given fetal thymic grafts eight weeks prior to infection to provide an endogenous $CD4^+$ T cell

population (**Fig. 7b,c**). Following infection with *Trichuris*, littermate control mice developed pathogen-induced Th2 cytokine responses characterized by production of IL-4, IL-5 and IL-13 by mesenteric lymph node (mLN) cells (**Fig. 8a**). In contrast, MHC II^{CD11c} mice exhibited minimal infection-induced production of Th2 cytokines (**Fig. 8a**). Histological analysis of intestinal tissues in infected control mice revealed hallmarks of type 2 inflammation including goblet cell hyperplasia as determined by expression of Gob5 (chloride channel calcium activated 3, mCLCA3) (**Fig. 8b**), a goblet cell-associated marker regulated by Th2 cytokines and linked with type 2 inflammation, (Zaph *et al.*, 2007) and increased mucin production assessed by Alcian blue/periodic acid-Schiff staining of cecal sections (**Fig. 8c**). Consistent with decreased Th2 cytokine production, infected MHC II^{CD11c} mice exhibited a marked absence of goblet cells and goblet cell-derived proteins (**Fig. 8b,c**). Th2 cytokine-dependent expression and luminal secretion of goblet cell-derived resistin-like molecule beta (RELMβ) in resistant mice provides a non-invasive indicator of the kinetics of Th2 cytokine responses in the intestinal microenvironment (Artis *et al.*, 2004b). As previously reported, luminal RELMβ levels peaked in resistant control mice between days 12 and 18 post-infection(Artis *et al.*, 2004b) (**Fig. 8d**), coincident with worm expulsion, while luminal secretion of RELMβ in infected MHC II^{CD11c} mice was dramatically reduced in magnitude (**Fig. 8d**). Associated with a polarized Th2 cytokine response, littermate control mice also displayed increased IgG1 and IgE (data not shown). However, since MHC II^{CD11c} mice lack MHC class II expression on B cells (Lemos *et al.*, 2003), no antigen-specific class-switched antibody was detected in infected MHC II^{CD11c} mice (data not shown). Critically, the defect in Th2 cytokine responses in MHC II^{CD11c} mice resulted in susceptibility to infection in mice on a normally genetically resistant background (**Fig. 8e**). Taken together, these data

demonstrate that restriction of MHC class II-dependent antigen presentation to $CD11c^+$ cells was insufficient to promote $CD4^+$ Th2 cell-dependent immunity following intestinal helminth infection.

2.4.2 Antigen presentation by $CD11c^+$ cells is sufficient to promote $CD4^+$ T cell proliferation and Th1 cell differentiation

The extensive physical and biochemical barriers separating antigenic material within the enteric space from lymphocytes in the underlying lymphoid follicles and lamina propria of the intestine create unique challenges in antigen sampling and presentation (discussed in **Chapter 1.1** and reviewed in (Artis, 2008)). Therefore the impaired Th2 cytokine responses in *Trichuris*-infected MHC II^{CD11c} mice may indicate that additional antigen-presenting cells are required for either the sampling of *Trichuris* antigens or the provision of signals necessary for the priming, expansion and differentiation of pathogen-specific $CD4^+$ Th2 cells. However, following *Trichuris* infection both littermate control mice and MHC II^{CD11c} mice exhibited an infection-induced increase in total $CD4^+$ T cell numbers in the draining mLN (**Fig. 9a**), suggesting that $CD11c^+$ DC-restricted antigen presentation is sufficient for the priming and expansion of $CD4^+$ T cells following intestinal infection. To determine whether $CD4^+$ T cells in infected MHC II^{CD11c} mice were non-responsive or had received signals for alternative differentiation, mRNA was isolated from mLN of naïve and infected control or MHC II^{CD11c} mice and analyzed for expression of IL-10, IL-17, and IFN-γ to assess the magnitude of Treg, Th17 and Th1 responses. While there was little to no induction of IL-10 or IL-17 mRNA expression in infected control and MHC II^{CD11c} mice, IFN-γ mRNA was selectively and significantly induced in infected MHC II^{CD11c} mice compared to controls (**Fig. 9b**). Consistent with elevated IFN-γ mRNA expression, the frequency of mLN $CD4^+$ T cells producing IFN-γ (**Fig. 9c; bold**) as well

as the amount of IFN-γ made per cell (**Fig. 9c; italics**) were increased in infected MHC II^{CD11c} mice compared to control mice. Secretion of IFN-γ was also significantly elevated following *in vitro* stimulation of mLN cells isolated from infected MHC II^{CD11c} mice compared to control mice (**Fig. 9d**). Thus, following intestinal infection, cognate interactions between antigen-specific CD4$^+$ T cells and CD11c$^+$ DC alone are sufficient to promote the priming and expansion of CD4$^+$ Th1 cells but are insufficient for the development of Th2 cytokine-dependent immunity.

2.4.3 Blockade of IFN-γ restores type 2 immunity in MHC II^{CD11c} mice

One explanation for the aberrant IFN-γ response in infected MHC II^{CD11c} mice is that MHC II-dependent interactions between CD4$^+$ T cells and other APCs are required to either actively promote Th2 cell differentiation or inhibit Th1 cell differentiation. Thus, in the absence of additional APC interactions, DC-derived signals such as IL-12 preferentially induce Th1 cell differentiation. To determine whether alterations in the cytokine milieu could restore immunity in MHC II^{CD11c} mice, *Trichuris*-infected MHC II^{CD11c} mice were treated with a monoclonal anti-IFN-γ blocking antibody during the course of infection. Consistent with previous findings (**Fig. 8a, 9d**) stimulated T cells isolated from the mLN of infected MHC II^{CD11c} mice exhibited a robust IFN-γ response with low levels of IL-4, IL-5 and IL-13 (**Fig. 10a**). Associated with the lack of Th2 cytokines, MHC II^{CD11c} mice showed decreased goblet cell responses and were susceptibile to *Trichuris* infection (**Fig. 10b,c**). In contrast, anti-IFN-γ treatment of MHC II^{CD11c} mice resulted in a significant reduction in IFN-γ production and the emergence of a Th2 cytokine response characterized by significantly increased IL-4, IL-5 and IL-13 production by mLN cells (**Fig. 10a**), goblet cell hyperplasia (**Fig. 10b**), and recovery of immunity to infection (**Fig.**

10c). In total, these data suggest that following blockade of a non-protective Th1 cytokine response, $CD11c^+$ cells can provide the antigen-specific interactions to drive $CD4^+$ Th2 cell differentiation and protective immunity. However, in the presence of endogenous IFN-γ signals, MHC class II expression on non-$CD11c^+$ cells is required for the development of protective Th2 cytokine responses *in vivo.*

2.4.4 $CD11c^+$ cells contribute to the optimal expansion of $CD4^+$ T cells but are not required for Th2 cytokine-dependent immunity

To determine the relative contribution of $CD11c^+$ cells in immunity to *Trichuris* we utilized CD11c-DTR mice that express the high-affinity simian diphtheria toxin (DT) receptor under control of the CD11c promoter. In this model system, littermate control transgene-negative mice remain non-responsive to DT injection while DT administration to CD11c-DTR mice results in the selective apoptosis of $CD11c^+$ cells (Jung *et al.*, 2002). To avoid the toxicity associated with long-term DT treatment of intact CD11c-DTR mice, we employed bone marrow (BM) chimeras of either WT or CD11c-DTR donor BM into WT recipients. Mice were infected with *Trichuris* and given DT injections every 72 hours for three weeks. Consistent with previous studies utilizing CD11c-DTR mice, transient depletion of $CD11c^+$ cells (**Fig. 11a**) throughout the course of *Trichuris* infection resulted in a significant reduction in mLN $CD4^+$ T cell numbers (**Fig. 11b**). However, unexpectedly, there was little effect on the expression of goblet cell-derived RELMβ (**Fig. 11c**), production of Th2 cytokines (**Fig. 11d**) or the course of infection (**Fig. 11e**). These data indicate that $CD11c^+$ cells may not be essential for protective immunity to *Trichuris* and that another APC may be required for the development of Th2 cytokine-dependent immunity *in vivo*.

2.4.5 Th2 cytokine-dependent immunity to *Trichuris* is independent of macrophages and B cells

In addition to DCs, macrophages and B cells are professional APCs implicated in the development of adaptive $CD4^+$ Th2 cell-dependent immunity. For example, macrophages have been shown to be critical in secondary immunity to the helminth *Heligomosoides polygyrus* (Anthony et al., 2006) but their role in the developing primary Th2 cytokine response to helminths is less clear. To examine the requirement of macrophages in primary immunity to *Trichuris*, we depleted macrophages using clodronate-loaded liposomes. While i.v. injection of clodronate-loaded liposomes efficiently depleted $CD11b^+$ cells (**Fig. 12a**), the depletion of macrophages throughout the course of *Trichuris* infection had no effect on the development of Th2 cytokine-dependent inflammation (**Fig. 12b**) or worm expulsion (**Fig. 12c**). B cells express constitutively high levels of MHC class II and a recently published study demonstrated that effector cytokine-producing "Be2" cells promote Th2 cell differentiation via MHC II-, IL-2- and TNFα-dependent mechanisms during *H. polygyrus* infection (Wojciechowski *et al.*, 2009). However, previous work demonstrated that adoptive transfer of $CD4^+$ T cells alone into mice lacking both B and T cells was sufficient to restore immunity to *Trichuris* (Else and Grencis, 1996) suggesting that B cells are not required for protective immunity. In support of these findings, mice deficient in B cells (μMT mice) (**Fig. 12d**) exhibited intact Th2 cytokine-dependent goblet cell responses (**Fig. 12e**) and expulsion of *Trichuris* (**Fig. 12f**). Thus, while macrophages and B cells likely contribute to type 2 immunity in a physiologic setting, they do not have essential non-redundant roles in host protective immunity to *Trichuris*.

2.5 Discussion

Dendritic cells are critical antigen presenting cells in the initiation of adaptive $CD4^+$ T cell responses and previous studies have shown that DCs are both necessary and sufficient to promote type 1 immune responses. For example, bone marrow-derived DCs pulsed with antigen in the context of various TLR ligands are sufficient to drive naïve $CD4^+$ T cell proliferation and Th1 cell differentiation (Schnare *et al.*, 2001) and, *in vivo,* DC-restricted MHC class II presentation is sufficient to promote functional $CD4^+$ Th1 cell-dependent immunity to the intracellular parasite *Leishmania major* (Lemos *et al.*, 2004). CD11c-DTR mice have also been used in a broad range of experimental models to demonstrate the requirement for DCs in type 1 immunity and inflammation. In these experiments, depletion of DCs led to defective type 1 immune responses following challenge with intracellular bacteria and viruses (Sapoznikov and Jung, 2008).

In contrast, the role of DCs in the initiation of type 2 immune responses has remained controversial, in part due to the lack of evidence for DC activation following exposure to helminth antigens. While stimulation of DCs via TLR ligation or pro-inflammatory cytokines results in their phenotypic maturation and promotion of Th1 cell differentiation, Th2 cell-promoting DCs or "DC2" are described as phenotypically immature, often expressing low levels of MHC class II and co-stimulatory molecules (MacDonald and Maizels, 2008). Consistent with this, we do not find evidence of changes in the surface phenotype of DCs isolated from the GALT following *Trichuris* infection (data not shown). In addition, while DC-derived IL-12 is a potent Th1 cell inducer, the search for DC-derived factors required to drive Th2 cell differentiation has been met with limited success. For example, while differential expression of the Notch ligand *jagged2* was

shown to promote Th2 cell differentiation *in vitro*, the requirement for *jagged2* expression was not replicated *in vivo* (Worsley *et al.*, 2008; Amsen *et al.*, 2004). Thus, current models of Th2 cell differentiation propose that after exposure to pathogens or allergens that elicit Th2 cell responses, DCs present antigens and promote T cell priming whilst production of IL-2 and IL-4 from either activated T cells themselves or innate immune cell populations, including eosinophils, mast cells and basophils, provide instructive signals for Th2 cell differentiation (**Fig. 13❶**).

Here, we demonstrate that MHC II-dependent antigen presentation by DCs is insufficient to drive protective type 2 immunity after helminth infection and that transient depletion of DCs does not alter the course of immunity, suggesting that DCs do not play a critical role in the development of Th2 cytokine-dependent immune responses. Taken together, our data suggests a revision of the current model that includes additional non-DC cognate MHC II-TCR interactions to initiate and/or maintain Th2 cell differentiation, whether via directed delivery of instructive Th2 polarization signals or active suppression of Th1 cell differentiation. One potential model is that, as with Th1 cell differentiation, $CD11c^+$ DCs are responsible for the priming and activation of naïve $CD4^+$ T cells within the lymph node. After initial priming the T cells then encounter another APC, either within the lymph node or at the site of infection, that provides the signals in trans to promote or maintain Th2 cell differentiation (**Fig. 13❷**). Alternatively, DCs may not act as the primary APC for pathogens or allergens that provoke Th2 cell responses. In this case, an unknown cell type may be involved in the innate recognition of the antigen, the priming of naïve $CD4^+$ T cells and the provision of signals to drive Th2 cell differentiation (**Fig. 13❸**). In both of these alternative models, autocrine IL-2 and IL-4 production by T

cells may act to amplify and stabilize the Th2 cell pool. Identifying the potential non-$CD11c^+$ DC cognate interactions required for the initiation and maintenance of Th2 cell–dependent immunity and inflammation could inform the design of new vaccine strategies for helminth infections and provide novel therapeutic targets in combating Th2 cytokine-dependent inflammation associated with allergy and asthma.

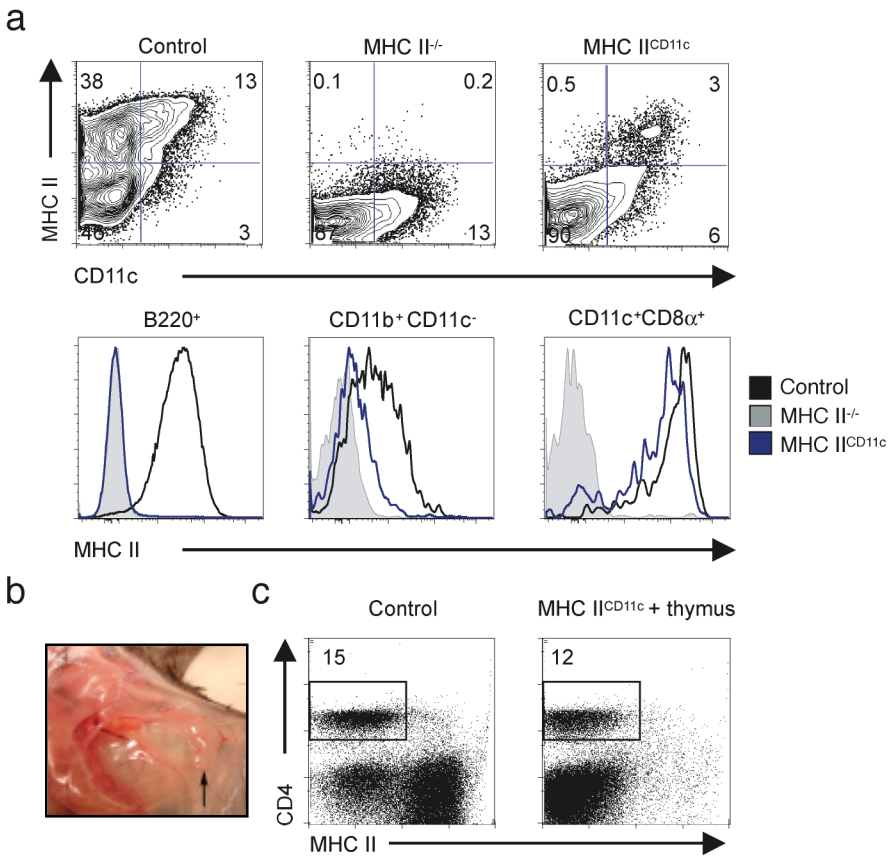

Figure 7. MHC class II expression is restricted to CD11c^{+} DC in MHC II^{CD11c} mice. (a) Flow cytometry of mLN cells from littermate control, MHC II$^{-/-}$ or MHC II^{CD11c} mice were stained for surface MHC class II and CD11c (upper panel) or MHC class II, B220, CD11b, CD11c and CD8α (lower panel). (b) Picture of engrafted thymus. Arrow indicates vascularized thymic graft. (courtesy of Eric Allenspach) (c) Flow cytometry of splenocytes demonstrating reconstitution of CD4^{+} T cells in MHC II^{CD11c} mice after thymic graft.

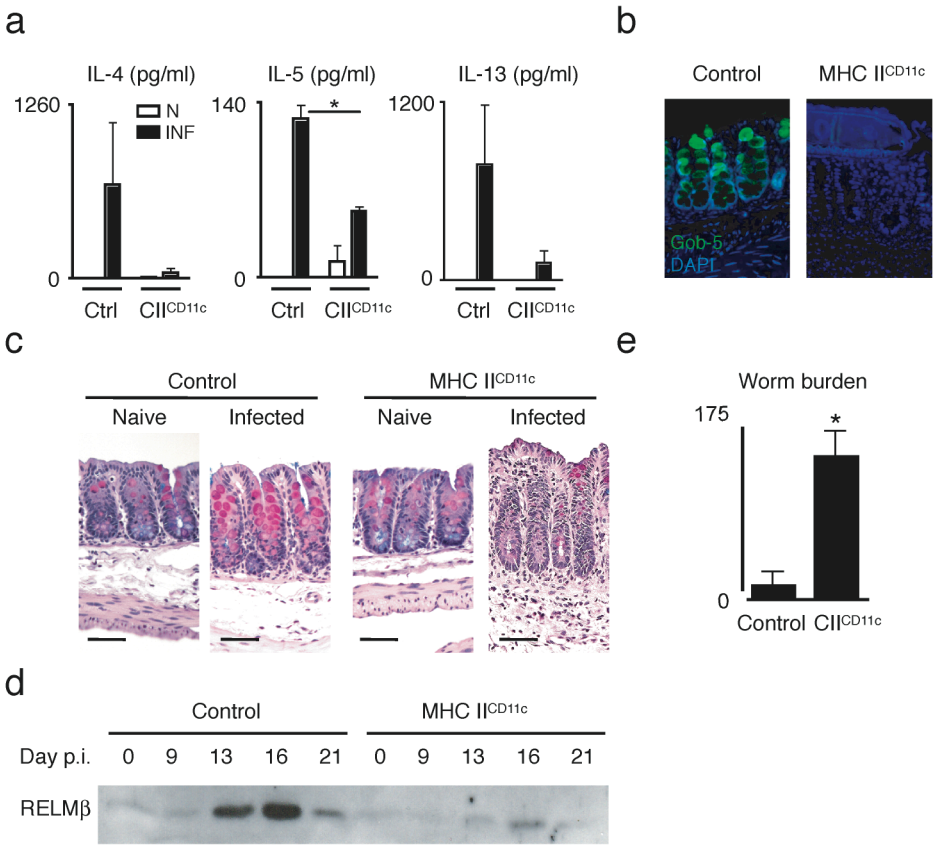

Figure 8. MHC class II expression restricted to CD11c^{+} DC is insufficient to promote type 2 immunity to intestinal helminth infection. (a-e) Littermate control and MHC II^{CD11c} mice were infected with *Trichuris* eggs and sacrificed on day 21 post-infection. (a) mLN cells from naive (N, open bars) and infected (INF, filled bars) mice were cultured *in vitro* for 48 hours and supernatants were assayed by ELISA for IL-4, IL-5, and IL-13 secretion. **P* = 0.003 (b) Cecal sections from infected control and MHC II^{CD11c} mice were stained by immunofluoresence for Gob-5 (green) and DAPI (blue). (c) Cecal sections from naive and infected control or MHC II^{CD11c} mice were stained with Alcian blue/periodic-acid Schiff reagent to detect mucins. (d) Protein extracted from

pooled fecal pellets of control and MHC II^{CD11c} mice collected on the indicated days post-infection was analyzed by Western blot to assess luminal secretion of RELMβ. (e) Cecal worm burdens from infected control and MHC II^{CD11c} mice were determined microscopically at day 21 post-infection. *P = 0.0006 Results are representative of three independent experiments with n = 3-5 mice per group; graphs represent mean +/- SEM.

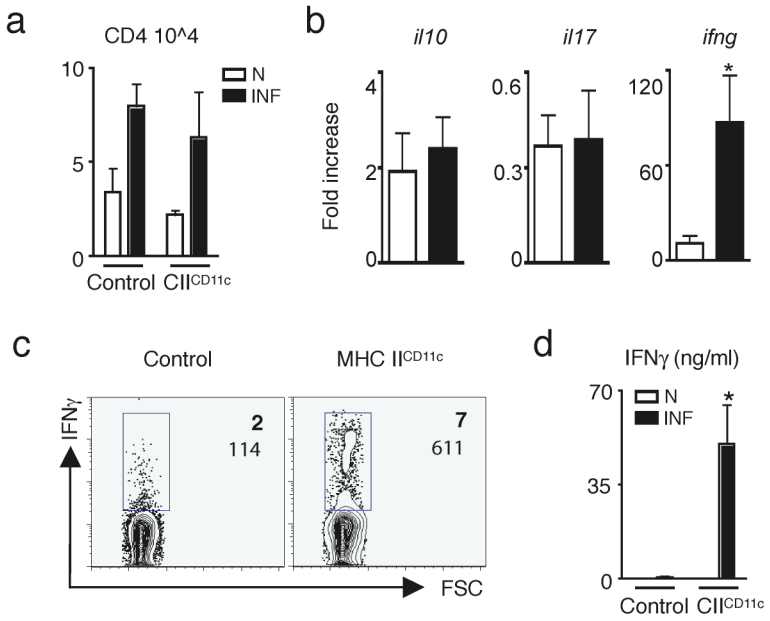

Figure 9. Intact proliferation and Th1 cell differentiation following *Trichuris* infection in MHC II^{CD11c} mice. (a) mLN cells from naive (N, open bars) or *Trichuris*-infected (INF, filled bars) littermate control and MHC II^{CD11c} mice were counted and total numbers of CD4$^+$ T cells determined by flow cytometry. (b) *il10, il17 and ifng* mRNA expression in mLN was determined by quantitative real-time PCR. Values represent mean +/-SEM of the fold-increase over naive controls. **P* = 0.03 (c) Flow cytometry of intracellular IFNγ staining of mLN cells from infected control or MHC II^{CD11c} mice. Plots are gated on CD4$^+$ T cells. Bold = %IFNγ$^+$, below = mean fluorescence intensity. (d) IFNγ secretion by mLN cells isolated from naive (N, open bars) and infected (INF, filled bars) mice was examined by ELISA. **P* = 0.03.

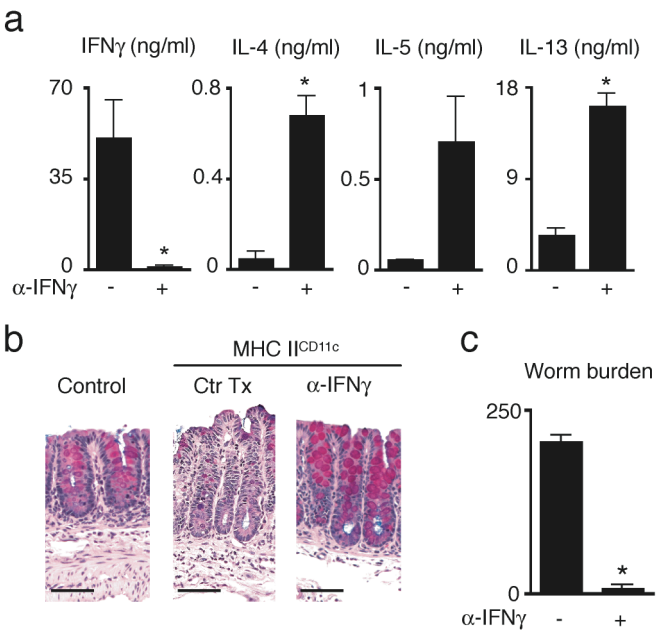

Figure 10. Blockade of IFNγ in MHC II^{CD11c} mice recovers Th2 cytokine-dependent immunity to *Trichuris* infection. (a) Cytokine production by stimulated mLN cells isolated from control-treated (Tx) or anti-IFNγ-treated MHC II^{CD11c} mice was examined by ELISA. IFNγ **P* = 0.03, IL-4 **P* = 0.002, IL-13 **P* = 0.001. (b) Cecal sections from littermate control, control-treated or anti-IFNγ treated MHC II^{CD11c} mice stained for mucins with Alcian blue/periodic-acid Schiff. (c) Worm burdens from infected control-treated or anti-IFNγ treated MHC II^{CD11c} mice at day 21 post-infection. **P* < 0.001. Results are presented as mean +/- SEM of 3-4 mice per group.

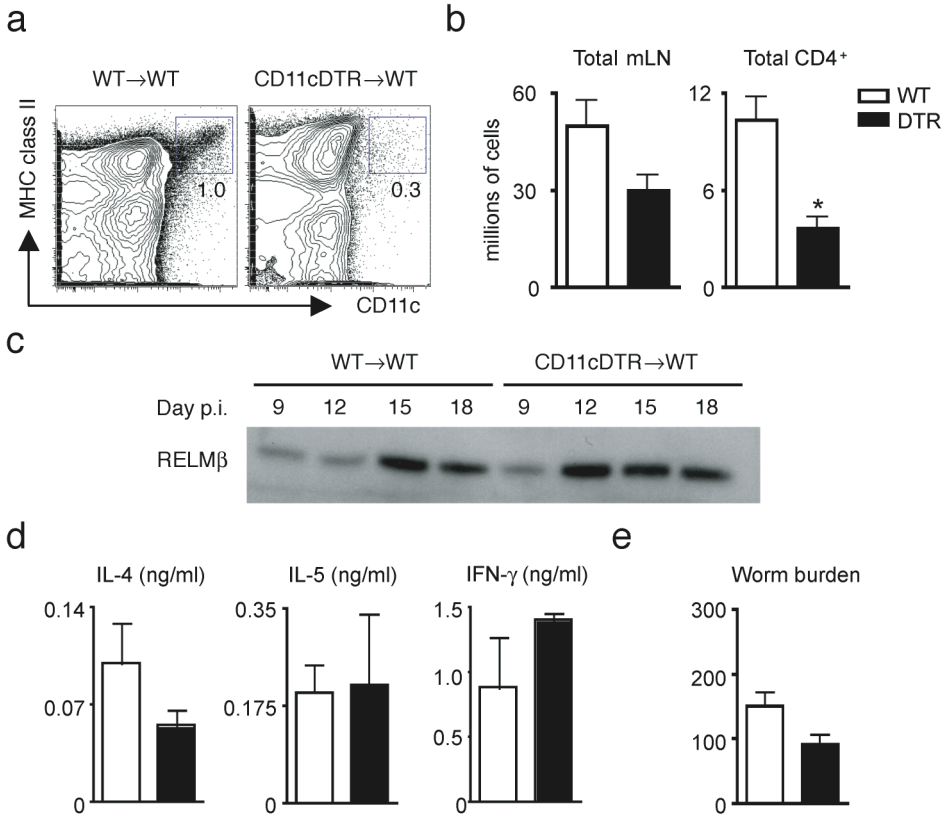

Figure 11. Depletion of CD11c$^+$ cells during *Trichuris* infection does not influence expression of Th2 cytokines or expulsion of *Trichuris*. (a) Flow cytometry of splenic DC day 2 following injection of 100ng diphtheria toxin i.p. in WT and CD11c-DTR mice (b) Total mLN cellularity and total numbers of mLN CD4$^+$ T cells at day 21 post-*Trichuris* infection in WT or CD11c-DTR chimeric mice depleted of DC every three days throughout infection. (c) Western blot of RELMβ from pooled fecal pellets. (d) ELISAs of supernatants from mLN cells from day 21 infected WT or CD11c-DTR chimeric mice polyclonally stimulated with anti-CD3/anti-CD28 for 48 hours. (e) Worm burdens at day 21 post-infection in WT or CD11c-DTR chimeric mice. Results are representative of 2 independent experiments with 2-4 mice per group.

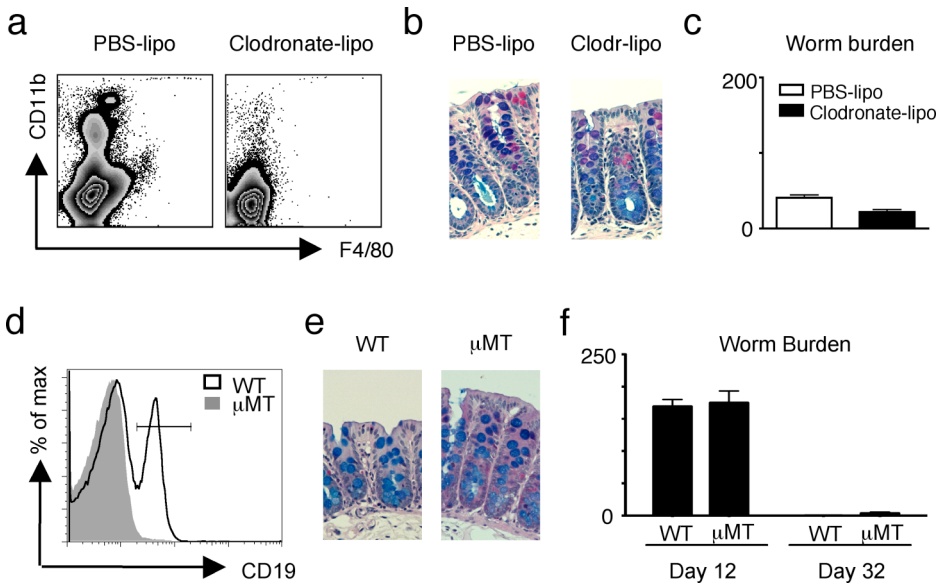

Figure 12. Th2 cytokine-dependent immunity to *Trichuris* is independent of macrophages and B cells. (a) Flow cytometry of splenocytes from mice treated with either control PBS loaded-liposomes or Clodronate-loaded liposomes. (b) Alcian blue/periodic acid-Schiff staining of cecal section from infected control or macrophage-depleted mice. (c) Cecal worm burdens at day 21 post-infection. (d) Flow cytometry of splenocytes from either WT (solid line) or μMT mice (filled grey). (e) Alcian blue/periodic acid-Schiff staining of cecal section from infected WT or μMT mice. (f) Worm burdens at day 12 and day 32 post-infection of WT or μMT mice. Data generated by Paul Giacomin (a-c) and Colby Zaph (d-f).

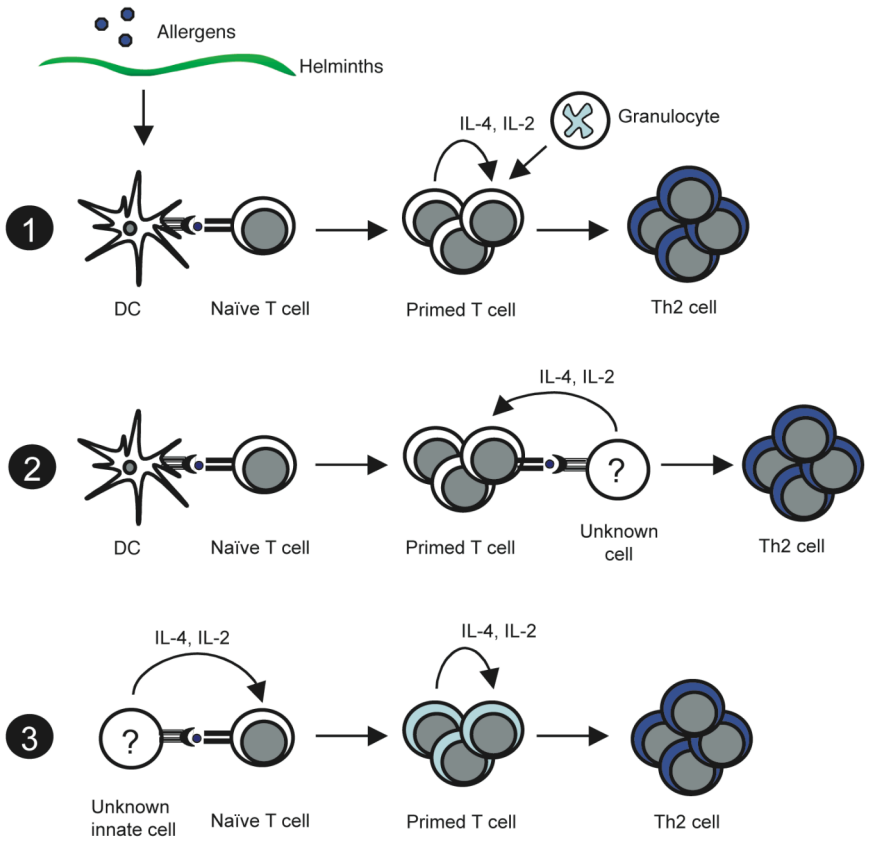

Figure 13. Models for Th2 cell differentiation following exposure to type 2 pathogens or allergens.

Chapter 3

MHC class II-dependent basophil-CD4$^+$ T cell interactions promote Th2 cell-dependent immunity to helminth infection

3.1 Abstract

Although dendritic cells (DCs) are the only antigen presenting cells (APCs) thought to prime naïve CD4$^+$ T cells, their role in priming Th2 cell responses has been unclear. Our previous studies (see **Chapter 2**) demonstrated that DC-restricted MHC class II expression was insufficient to drive Th2 cytokine-dependent immunity *in vivo,* provoking the search for additional APCs that could provide both cognate MHC II-TCR interactions and signals in *trans* to promote Th2 cell differentiation. Using IL-4/eGFP reporter mice, we identified basophils as a dominant innate immune cell population that co-expressed MHC class II and *il4* message following infection with the intestinal helminth *Trichuris*. Basophilia was promoted by thymic stromal lymphopoietin (TSLP) and depletion of basophils impaired protective immunity to helminth infection. *In vitro*, basophils promoted antigen-specific CD4$^+$ T cell proliferation and IL-4 production in an MHC class II-dependent manner and adoptive transfer of basophils augmented the expansion of helminth-responsive CD4$^+$ T cells *in vivo*. Collectively, these studies suggest that MHC class II-dependent cognate interactions between basophils and CD4$^+$ T cells can promote optimal Th2 cytokine responses and protective immunity against helminth infection.

3.2 Introduction

The recruitment and activation of mast cells, eosinophils, and basophils are hallmarks of Th2 cytokine-dependent inflammation in peripheral tissues. While their roles in effector responses, including the release of inflammatory mediators such as histamine, leukotrienes, and interleukins, are well characterized (Prussin and Metcalfe, 2003), recent evidence suggests that these granulocyte populations can also function as accessory cells in the initiation of $CD4^+$ Th2 cell responses. For example, mast cells, eosinophils, and basophils are competent to produce and secrete IL-4 from intracellular stores, implicating these populations as early sources of IL-4 that could promote $CD4^+$ Th2 cell differentiation (Gessner *et al.*, 2005; Mohrs *et al.*, 2005; Min *et al.*, 2004). In addition, mast cells and eosinophils can express MHC class II, and eosinophils have been implicated as potential antigen presenting cells in both airway inflammation and helminth infection (Padigel *et al.*, 2007; Skokos *et al.*, 2003; MacKenzie *et al.*, 2001). Consistent with an immunoregulatory role, eosinophils recruit $CD4^+$ Th2 cells to the lung during airway inflammation through their production of CCL17 and CCL22 (Jacobsen *et al.*, 2008).

The frequency of basophils is also increased following exposure to allergens and helminth parasites and recent studies have demonstrated that they are a dominant source of IL-4/IL-13 following helminth infection and contribute to protective immunity (Ohnmacht and Voehringer, 2008; Voehringer *et al.*, 2006; Voehringer *et al.*, 2004a). Although basophilia is a common feature of Th2 cytokine-mediated inflammation, little is known about how these cells are activated and recruited to peripheral tissues. Medzhitov and colleagues recently posited a conserved mechanism for basophil-

mediated recognition of parasite products and allergens through protease-dependent activation (Sokol *et al.*, 2008). In that study, basophils were recruited to the draining lymph node early following allergen exposure and were essential for the generation of Th2 cytokine responses elicited following papain immunization. However, the potential accessory cell functions of basophils during $CD4^+$ Th2 cell development remain unknown.

In this chapter, basophils were identified as a dominant accessory cell population that co-expressed *il4* message and MHC class II following *Trichuris* infection, and *in vitro* studies showed that basophils could promote MHC class II-dependent antigen-specific $CD4^+$ T cell proliferation and Th2 cell differentiation. Moreover, *in vivo* depletion of basophils resulted in impaired protective immunity to *Trichuris* and adoptive transfer of primary WT basophils augmented $CD4^+$ T cell proliferation in response to *Schistosoma mansoni* egg injection. Taken together, these data suggest a previously unrecognized role for basophils in MHC class II-dependent cognate interactions with $CD4^+$ T cells that promote parasite-specific Th2 cytokine responses and host protective immunity.

3.3 Methods

3.3.1 Mice and parasites

6-8 week-old C57BL/6 mice were ordered from The Jackson Laboratories (Bar Harbor, ME). 4-get/IL-4eGFP (from Markus Mohrs, Trudeau Institute) and DO11.10 mice were bred and housed in specific pathogen-free conditions at the University of Pennsylvania. All experiments were performed following the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. *Trichuris* was maintained in

genetically susceptible mouse strains and eggs harvested as previously described (Zaph *et al.*, 2006). Briefly, infected susceptible mice were sacrificed at day 34 post-infection and adult worms isolated from the ceca and placed in RPMI with 5X penicillin/streptomycin. Media collected at 4 hours was dialyzed 3 times against PBS and filtered for use as antigen. Eggs secreted by adult worms were collected from media after both 4 and 24 hours of culture, washed with water, filtered over a 70μm cell strainer and allowed to embryonate in the dark for six weeks. Mice were infected by oral gavage with 200-300 embryonated *Trichuris* eggs. *Schistosoma mansoni* eggs, prepared as previously described (Taylor *et al.*, 2006), were provided by Edward Pearce's lab. Mice were injected in the hind footpads with 2500 eggs in 50μl PBS.

3.3.2 Polyclonal T cell stimulation

mLN were harvested and single-cell suspensions prepared in complete media (DMEM supplemented with 10% heat-inactivated FBS, 2mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25mM HEPES, and 5 x 10^{-5} M beta-mercaptoethanol). mLN cells were seeded in 48-well plates at 2.5x10^{6} per well and incubated with either media or 1μg/ml soluble anti-CD3/anti-CD28 (eBioscience) for 48 hours. Cell-free supernatants were harvested and cytokine production determined by sandwich ELISA. All antibody pairs purchased from eBioscience: clones AN-18, R4-6A2 (IFN-γ), 11B11, BVD6-24G2, (IL-4), TRFK5, TRFK4 (IL-5) and eBio13A, eBio 1316H (IL-13).

3.3.3 Western blots

Fecal protein isolation was performed as previously described (Artis *et al.*, 2004b). 30 μg of protein was loaded per sample for analysis by SDS-PAGE and immunoblotted for RELMβ using a polyclonal rabbit α-murine RELMβ antibody (Peprotech, Rocky Hill, NJ).

3.3.4 Real-time quantitative PCR

RNA was isolated from intestinal tissues of mice using a TRizol extraction (Invitrogen) and from mLN cells using RNEasy Spin Columns (Qiagen). Tissues were disrupted in a tissue homogenizer (TissueLyzer, Qiagen) and cDNA synthesized using Superscript Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was carried out on cDNA samples using commercial primer sets (Qiagen) and SYBR Green chemistry. All reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Samples are normalized to naïve controls unless otherwise stated.

3.3.5 Histology and immunofluoresence

Cecal tips were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. 5 μm sections were cut and stained for hematoxylin and eosin (H&E) or Alcian blue/periodic acid-Schiff. Unstained sections prepared on immunoslides were stained for Gob5 by immunofluoresence as previously described (Zaph *et al.*, 2007). Briefly, samples were de-paraffinized by consecutive methanol/ethanol rinses, boiled in citric acid buffer and stained with an antibody against Gob5 at 4 degrees overnight followed by staining with a Cy2-conjugated anti-goat antibody. For imaging of MHC II and IL-4-eGFP on basophils, 10 4-get mice were injected with 2500 *S. mansoni* eggs each in the hind footpads. Popliteal lymph nodes were harvested 2 days later and single cell suspensions prepared. Lymph node cells were positively selected for CD49b expression by MACs column purification (Milltenyi) and stained with fluorochrome-conjugated monoclonal antibodies against B220, CD3, c-kit, CD49b, and FcεRI (BD Bioscience and eBioscience). Basophils were sorted based on negative staining for B220, CD3 and c-Kit, positive staining for CD49b, FcεRI and expression of IL-4/eGFP using a FACS Aria (BD Bioscience). Sorted basophils were then stained in a FACs tube with biotinylated

anti-MHC class II (M5/114) followed by streptavidin-Cy2 for 20 minutes on ice, washed in FACs buffer and fixed in 2% PFA 10 minutes on ice. Stained basophils were then subjected to cytospin, washed with PBS and nuclei stained with DAPI. Slides were coverslipped and analyzed by 2-photon confocal microscopy.

3.3.6 Antibody and recombinant cytokine treatment

Basophils were depleted by i.p. injection of 10μg anti-FcεRI (MAR-1, eBioscience) on days 0,1,2 and 10, 11, 12 post-infection. Recombinant murine IL-25 (4μg/ml), IL-33 (20μg/ml), and TSLP (0.1mg/ml) were all purchased from RnD Systems and 100μl in PBS was injected i.p. once daily for four days.

3.3.7 Basophil isolation and $CD4^+$ T cell co-culture

$CD4^+$ T cells were isolated from spleens by negative selection via incubation with hybridoma supernatants (αB220, $\alpha F_C R$, αCD8, αMHCII) followed by magnetic bead purification (Qiagen). To obtain purified basophils, blood, spleen, and mLN cells were isolated from 4-get IL-4/eGFP reporter mice injected with 10μg rTSLP (RnD System) i.p. once daily for 4 days to enrich for basophils, positively selected for CD49b expression by MACs column purification (Milltenyi) and stained with fluorochrome-conjugated monoclonal antibodies against B220, CD3, c-kit, CD49b, and FcεRI (BD Bioscience and eBioscience). Basophils were sorted based on negative staining for B220, CD3 and c-Kit, positive staining for CD49b, FcεRI and expression of IL-4/eGFP using a FACS Aria (BD Bioscience). Following purification, sorted basophils were resuspended at $1x10^5$ cells per ml in complete medium. 100μl of basophils were used for cytospin and stained by Diffquick to confirm cellular morphology. Between $5x10^3$ and $1x10^4$ basophils were co-cultured with $2x10^5$ purified, CFSE-labeled DO11.10 $CD4^+$ T cells with 10ng per ml

rIL-3 (RnD Systems) in the presence or absence of 1μg per ml OVA peptide and 5μg/ml blocking antibody for MHC class II (M5/114). After four days of culture, cells were stimulated with PMA, ionomycin, and brefeldin A for 4 hours. Cells were pelleted at 1500 RPM for 5 min. Supernatants were collected for ELISA and cells washed in FACS buffer, incubated with Fc Block (2.4G2 and rat IgG) for 10 min on ice, stained with fluorochrome-conjugated monoclonal antibodies against CD4 and fixed with 2% paraformaldehyde. Cells were permeabilzed with 0.4% saponin in FACS buffer and stained for intracellular cytokines using fluorochrome-conjugated monoclonal antibodies against IL-4 and IL-13 (eBioscience).

3.3.8 Basophil transfer

C57BL/6 mice were injected with 2.5 x 10^3 *S. mansoni* eggs in each footpad, popliteal lymph nodes, spleen, and blood pooled two days later, and basophils purified by sequential CD49b enrichment and cell sorting as described above. Recipient MHC II^{CD11c} mice were given 1 x 10^7 purified CFSE-labeled $CD4^+$ T cells from naïve C57BL/6 mice one day prior to egg injection. Sorted basophils were resuspended in a PBS/*S. mansoni* egg suspension and each recipient MHC II^{CD11c} mouse given either 5 x 10^4 basophils and 2.5 x 10^3 *S. mansoni* eggs or 2.5 x 10^3 *S. mansoni* eggs alone in the right hind footpad in a volume of 50μl. Draining and non-draining pLN cells were isolated 4 days post-egg injection, stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry on a FACSCanto II (BD Biosciences).

3.3.9 Statistics

Results represent the mean ± SEM unless otherwise stated. Statistical significance was determined by the Student's *t* test.

3.4 Results

3.4.1 Basophils co-express MHC class II and IL-4 mRNA

In **Chapter 2**, we demonstrated that antigen presentation by $CD11c^+$ DCs alone was insufficient to promote Th2 cell-dependent immunity following infection with *Trichuris* and that in the absence of additional cognate MHC class II-TCR interactions, a non-protecitve Th1 cell response developed. Additionally, we found that neither B cells nor macrophages played essential roles in immunity to infection. We therefore focused on the identification of innate immune cells that could both express MHC class II and provide an innate source of IL-4 following *Trichuris* infection. We previously employed IL-4/eGFP (4-get) reporter mice to track emerging $CD4^+$ Th2 responses following *Trichuris* infection (Zaph *et al.*, 2006). 4-get mice contain an internal ribosomal entry site (IRES)-enhanced green fluorescent protein (eGFP) element within the IL-4 locus allowing direct *ex-vivo* analysis of cells competent to express IL-4 (Mohrs *et al.*, 2001). We utilized the same *in vivo* approach to identify non-B non-T cells that co-expressed IL-4 mRNA and MHC class II. Gating on non-B non-T cells, we identified a cell population that was IL-4/$eGFP^+$ (**Fig. 14a**) and expressed MHC class II (**Fig. 14b**). Previous studies have shown that mast cells and eosinophils can express MHC class II (Padigel *et al.*, 2007; Skokos *et al.*, 2003; MacKenzie *et al.*, 2001) and are competent to produce IL-4 (Gessner *et al.*, 2005; Mohrs *et al.*, 2005). However, classical mast cells (c-kit^+ SSC^{high}) were not found following infection with *Trichuris* and frequencies of SIGLEC-F^+ SSC^{high} eosinophils were decreased after infection (**Fig. 14c**). In contrast, $CD49b^+$ $Fc\varepsilon RI^+$ basophils emerged as a dominant cell population expressing both IL-4 mRNA and MHC class II following *Trichuris* infection (**Fig. 14c**), consistently comprising 40% of IL-4/$eGFP^+$ MHC class II^+ cells. Basophils expressed intermediate levels of MHC class II

compared to MHC class II-deficient basophils (**Fig. 14d**). While there have been previous reports of MHC class II expression on eosinophils (Padigel *et al.*, 2007; MacKenzie *et al.*, 2001), this is the first report we are aware of demonstrating MHC class II expression on murine basophils and suggests a potential accessory cell function for this cell population during helminth infection.

3.4.2 *In vivo* depletion of basophils impairs protective immunity to *Trichuris*

To determine whether basophils play a role in the development of Th2 cytokine-dependent protective immunity, WT C57BL/6 mice were infected with *Trichuris* and treated with either control Ig or a monoclonal antibody against the FcεRI (MAR-1). Previous studies have demonstrated efficient depletion of basophils for up to 10 days following i.p. injection of MAR-1 (Denzel *et al.*, 2008) and we observed greater than 90% depletion of basophils at day 21 post-infection (**Fig. 15a**) following MAR-1 treatment. Depletion of basophils in infected mice resulted in decreased IL-4 mRNA expression (**Fig. 15b**), a marked reduction in Th2 cytokine-dependent goblet cell hyperplasia (**Fig. 15c**) and a decrease in luminal secretion of RELMβ in the intestine (**Fig. 15d**). Loss of basophils and impaired Th2 cytokine responses were associated with impaired expulsion of *Trichuris* (**Fig. 15e**). Taken together, these data support a role for basophils in the development of protective type 2 immunity to intestinal helminth infection.

3.4.3 TSLP selectively elicits basophils

We recently identified essential functions for intestinal epithelial cell (IEC)-derived cytokines IL-25 (Owyang *et al.*, 2006) and TSLP (Zaph *et al.*, 2007) in the development of Th2 cytokine-dependent immunity to *Trichuris.* In addition, Grencis and colleagues identified a role for IEC-derived IL-33 in promoting Th2 cytokine responses and worm

expulsion (Humphreys *et al.*, 2008) and several studies have demonstrated that IL-33 treatment can directly stimulate cytokine and chemokine production from basophils and mast cells *in vitro* (Pecaric-Petkovic *et al.*, 2009; Smithgall *et al.*, 2008; Suzukawa *et al.*, 2008). To test whether IL-25, IL-33, or TSLP contributed to basophil responses *in vivo*, 4-get mice were injected with recombinant IL-25, IL-33, and TSLP and the peripheral basophil responses examined by flow cytometry. As previously reported, IL-25 treatment elicited a robust population of IL-4/eGFP^{+} SSC^{high} cells(Fallon *et al.*, 2006) (**Fig. 16a**). Treatment with IL-33 also resulted in a marked elevation in the frequency of of IL-4/eGFP^{+} SSC^{high} cells (**Fig. 16a**). However, phenotypic analysis of these IL-4/eGFP^{+} cells revealed two distinct cell populations selectively elicited by each cytokine. IL-25 treatment resulted in increased frequencies of a non-B non-T c-kit^{+} mast cell-like population while IL-33 treatment led to increases in the frequency of CCR3^{+} eosinophils (data not shown). While administration of TSLP also resulted in a 3-fold increase in IL-4/eGFP^{+} cells over PBS-treated controls (**Fig. 16a**), unlike IL-25 and IL-33, TSLP treatment selectively elicited CD49b^{+} FcεRI^{+} basophils (**Fig. 16b).** These data suggest that while IEC-derived IL-25, IL-33 and TSLP promote expansion of diverse innate cell populations competent to produce IL-4, only TSLP promotes basophil expansion.

3.4.4 Basophils promote CD4^{+} Th2 cell differentiation *in vitro*

Demonstration that depletion of basophils resulted in impaired immunity to *Trichuris* (**Fig. 15**) coupled with the co-expression of MHC class II and *Il4* mRNA (**Fig. 14**) suggested that they may participate in MHC class II-dependent cognate interactions with CD4^{+} T cells to promote Th2 cell differentiation. To test whether basophils could present antigen via MHC class II, an *in vitro* co-culture system was adopted in which antigen-pulsed purifed basophils were co-cultured with purified CFSE-labeled ovalbumin (OVA)-

specific DO11.10 $CD4^+$ T cells. Sorted basophils exhibited characteristic multi-lobed nuclei and expressed both MHC class II and IL-4/eGFP (**Fig. 17a**). While minimal proliferation was detected in the absence of OVA peptide, approximately 75% of $CD4^+$ T cells co-cultured in the presence of antigen-pulsed basophils had diluted CFSE, consistent with proliferation (**Fig. 17b**). Basophil-induced $CD4^+$ T cell proliferation was dependent on MHC class II expression as addition of a blocking antibody against MHC class II abrogated these responses (**Fig. 17b**). To determine whether basophils could influence $CD4^+$ Th2 cell differentiation following antigen-specific stimulation of T cells, intracellular cytokine staining for IL-4 was performed (**Fig. 17b**) and IL-4 secretion measured in supernatants from co-cultured cells (**Fig. 17c**). Supernatants from basophil-T cell co-cultures in the absence of antigen contained basal amounts of IL-4 (**Fig. 17c**) and upon addition of OVA peptide, there was a 2- to 3-fold increase in secreted IL-4 that was abrogated in the presence of anti-MHC class II (**Fig. 17c**). Therefore, MHC class II-dependent cognate interactions between basophils and $CD4^+$ T cells can promote antigen-specific Th2 cell differentiation *in vitro*.

3.4.5 Basophils are recruited to the lymph node and express MHC class II following exposure to *Schistosoma mansoni* eggs

We sought to determine whether the recruitment of IL-4/$eGFP^+$ MHC class II^+ basophils was unique to *Trichuris* infection or whether they were common events following exposure to other helminth parasites. To test this, we employed footpad injection of *S. mansoni* eggs whereby delivery of *S. mansoni eggs* results in an acute and synchronous Th2 cytokine responses in the draining popliteal lymph node (pLN). In previous studies we have demonstrated robust expansion of $CD4^+$ T cells following egg injection with

greater than 40% of pLN $CD4^+$ T cells becoming $BrdU^+$ and 20% expressing IL-4-eGFP (Taylor *et al.*, 2006) providing a powerful *in vivo* model to examine helminth-induced innate and adaptive responses. *S. mansoni* eggs were delivered into the footpad of 4-get mice and pLN harvested at various time points post-injection. Strikingly, we identified a transient recruitment of basophils to the draining pLN on day 2 post-injection with a greater than 20-fold increase in frequency (**Fig. 18a**) and number (**Fig. 18b**) that was absent by day 5 (data not shown). Sorted IL-4/$eGFP^+$ basophils from *S. mansoni* egg-injected mice exhibited characteristic multi-lobular nuclei by cytospin (**Fig. 18c**), were $Fc\varepsilon RI^+$, and expressed MHC class II by both flow cytometry (**Fig. 18d**) and immunofluoresence (**Fig. 18e**). We next investigated whether helminth-elicited basophils could influence $CD4^+$ T cell proliferation *in vivo*. To address this, CFSE-labeled $CD4^+$ T cells were adoptively transferred into MHC II^{CD11c} mice in which basophils are present but MHC class II negative due to lack of expression of CD11c. Sorted WT basophils from *S. mansoni* egg-injected mice were adoptively transferred into naïve MHC II^{CD11c} recipients (**Fig. 19a**). In earlier studies we found that unlike adoptively transferred T cells in $RAG^{-/-}$ recipient mice, donor $CD4^+$ T cells delivered into MHC II^{CD11c} mice do not undergo homeostatic proliferation, likely due to the fact that MHC II^{CD11c} mice have a normal $CD8^+$ T cell compartment (Allenspach *et al.*, 2008; Lemos *et al.*, 2003). In addition, we have previously observed that in the absence of additional antigen stimulation in recipient mice, transfer of basophils alone does not induce the recruitment of antigen-specific T cells to the pLN. We therefore challenged MHC II^{CD11c} mice that had received both T cells and basophils with *S. mansoni* eggs in the footpad. Following egg injection, MHC II^{CD11c} mice that received eggs alone exhibited a four- to five-fold increase in total $CD4^+$ T cell numbers in the draining pLN compared to the non-

draining pLN (**Fig. 19b**) and 50% of pLN $CD4^+$ T cells were CFSE-dim (**Fig. 19c**). In contrast, expansion of $CD4^+$ T cells was substantially augmented in MHC II^{CD11c} mice that had received activated basophils. At day 4 following delivery of *S. mansoni* eggs, there was a greater than 14-fold increase in total pLN $CD4^+$ T cells (**Fig. 19b**) and nearly 70% of $CD4^+$ T cells were CFSE-dim (**Fig. 19c**). The transfer of WT basophils also selectively augmented egg-induced IL-4 mRNA expression in the pLN over mice given eggs alone (**Fig. 19d**). Collectively, these data demonstrate that MHC class II^+ basophils are rapidly recruited to the LN following exposure to helminth antigens and can augment $CD4^+$ T cell proliferation, suggesting potential cooperation between basophils and DC in the efficient expansion of helminth-responsive $CD4^+$ T cells *in vivo*.

3.5 Discussion

In **Chapter 2**, we demonstrated that antigen presentation by $CD11c^+$ DCs alone was insufficient to promote $CD4^+$ Th2 cytokine-dependent immunity and inflammation following *Trichuris* infection, suggesting that an additional MHC class II-dependent APC-T cell interaction was necessary to mediate Th2 cell-dependent immunity. However, depletion studies or genetic deletion of macrophages and B cells revealed non-essential roles for these professional APC populations in protective immunity to *Trichuris*. In this chapter, we identify a previously unknown role for basophils as APCs following exposure to helminths. Basophils were identified as a dominant cell population that could co-express MHC class II and IL-4 mRNA following *Trichuris* infection and depletion of basophils *in vivo* resulted in impaired immunity to infection. Consistent with a potential accessory cell function, basophils were able to promote MHC class II-dependent antigen-specific Th2 cell proliferation and differentiation *in vitro*. Additionally, MHC class

II^+ basophils were rapidly recruited to the lymph node following exposure to *S. mansoni* eggs and augmented helminth-induced $CD4^+$ T cell proliferation *in vivo*.

Basophils are rare circulating cells that make up less than 0.5% of total blood leukocytes yet are evolutionarily conserved across all vertebrate species and can expand and accumulate in peripheral tissues in multiple type 2 inflammatory settings, including following exposure to allergens or helminth infections. Although basophils were first described by Ehrlich 130 years ago (Ehrlich, 1879), their scarcity, coupled with a lack of reagents with which to identify and specifically manipulate them, has made it difficult to study basophil function *in vivo*. However, recent advances in available surface markers and depleting antibodies have revealed distinct non-redundant roles for basophils in the initiation and maintenance of chronic allergic inflammation (Obata *et al.*, 2007), in augmenting $CD4^+$ Th2 cytokine responses (Sokol *et al.*, 2008; Oh *et al.*, 2007) and providing B cell help for IgE class-switching (Gauchat *et al.*, 1993) and enhanced humoral immune responses (Denzel *et al.*, 2008). To these functions, the results of the present study add a previously unappreciated role for basophils as accessory cells that can promote $CD4^+$ Th2 cell differentiation in part through MHC class II-dependent cognate interactions.

A critical question that emerges from these findings is where functional basophil-T cell cognate interactions occur *in vivo*. Basophils are readily found in the blood and spleen but have rarely been found within lymph nodes, where $CD4^+$ T cell priming is likely to take place. However, recent work from Medzhitov and colleagues demonstrating that basophils are transiently recruited to draining lymph nodes following exposure to

allergen (Sokol *et al.*, 2008), coupled with our work demonstrating recruitment of basophils to the lymph node following exposure to *S. mansoni* eggs, suggests that basophils have the capacity to directly interact with naïve T cells in peripheral lymphoid tissues. Alternatively, basophils may not be involved in initial priming of naïve $CD4^+$ T cells but act as accessory cells at the site of inflammation, where activated T cells may require additional cognate interactions to promote or maintain Th2 cell differentiation and effector function. Supporting this, a previous report utilizing cytokine reporter mice demonstrated that cytokine mRNA and protein expression are uncoupled following priming and expansion of naïve T cells, suggesting that additional activation at the site of infection may be required to license effector function (Scheu *et al.*, 2006).

Both eosinophils and basophils are short-lived cells that circulate in the blood, are present in low levels in the lung and intestine of naïve mice, and are recruited to sites of inflammation. However, while both cell types are prominent populations within inflamed tissues, evidence from multiple animal disease models suggests that the recruitment of these cells may be differentially regulated. For example, basophil recruitment to the lung is STAT6-independent but T cell-dependent while eosinophil recruitment requires both signals following helminth infection (Voehringer *et al.*, 2004a). Additionally, depletion studies with basophils suggest that these cells may provide chemotactic factors, either directly or indirectly, that are required for the recruitment of eosinophils (Brown *et al.*, 1982) and indicates that basophils may help to initiate and maintain chronic inflammatory responses. Microarray analyses of basophils sorted from the lung during *N. brasiliensis* infection also revealed high expression levels of the chemokines CCL3 (MIP1α), CCL4, (MIP1β), CCL6 (C10), and CCL17 (TARC), supporting a role for

basophils in the recruitment of activated $CD4^+$ T cells to the site of infection(Voehringer *et al.*, 2004a). Therefore, identifying the factors that regulate basophil expansion and recruitment could be an important target for modulating early events in the generation of $CD4^+$ Th2 cell-dependent immunity and inflammation.

Previous reports have identified T cell-derived IL-3 as a critical cytokine for basophilia during intestinal helminth infection (Shen *et al.*, 2008). However, whether other innate cell-derived cytokines contribute to early basophil responses is unclear. We recently identified a critical role for intestinal epithelial cell (IEC) activation in the generation of protective immunity to *Trichuris*. TSLP is an epithelial cell-derived cytokine implicated in promoting type 2 inflammation in the skin and lung through effects on both innate and adaptive immune cells and is also known to activate basophils (Zhou *et al.*, 2005). Here, we identify a novel role for TSLP as a soluble factor able to induce peripheral basophilia. In addition to TSLP, the IEC-derived cytokines IL-25 and IL-33 have also been implicated in the promotion of type 2 inflammation and in the activation of basophils (Pecaric-Petkovic *et al.*, 2009; Saenz *et al.*, 2008; Smithgall *et al.*, 2008; Suzukawa *et al.*, 2008; Allakhverdi *et al.*, 2007a; Angkasekwinai *et al.*, 2007; Owyang *et al.*, 2006; Schmitz *et al.*, 2005). However, we demonstrate that these cytokines, although capable of promoting expansion of IL-4/$eGFP^+$ innate cells, do not promote basophilia *in vivo*. The influence of TSLP, IL-25, IL-33 and other stimuli such as IL-3, IL-18, TLR ligation, and FcεRI cross-linking on basophil cytokine production, recruitment to LN, and APC function remains to be determined. In addition to IEC-derived cytokines, there is growing evidence to suggest that basophils can be directly activated by helminth-derived products and allergens that may act as “superallergens” to stimulate FcεRI cross-linking

in a non antigen-specific manner (Falcone *et al.*, 2001). For example, IPSEα1, a glycoprotein derived from *S. mansoni* eggs, has been shown to directly stimulate the production of IL-4 from basophils by an IgE-dependent but non antigen-specific mechanism (Schramm *et al.*, 2007). Thus, a combination of non-hematopoetic and innate immune cell-derived cytokines coupled with direct stimulation by helminth products or allergens may act together to elicit basophil expansion and activation *in vivo.*

In addition to the identification of a role for basophils in MHC class II-dependent promotion of Th2 cell differentiation and immunity to *Trichuris* infection, Medzhitov and colleagues have identified a critical role for basophils in the development of allergen-specific Th2 cytokine responses. In those studies, allergen-stimulated basophils expressed MHC class II, CIITA, the invariant chain and co-stimulatory machinery and promoted allergen-specific $CD4^+$ Th2 cell differentiation (Sokol *et al.*, 2009). In parallel, another group has shown that basophils can efficiently take up, process and present antigen-IgE complexes to drive Th2 cell differentiation both *in vitro* and *in vivo* (Yoshimoto *et al.*, 2009). Taken together, our findings indicate that basophil-mediated innate recognition of helminth-derived products, allergens and IgE-antigen complexes coupled with their MHC class II-dependent promotion of Th2 cell responses (**Fig. 20**) may be an evolutionarily conserved pathway that plays a cardinal role in the development of type 2 immunity and inflammation.

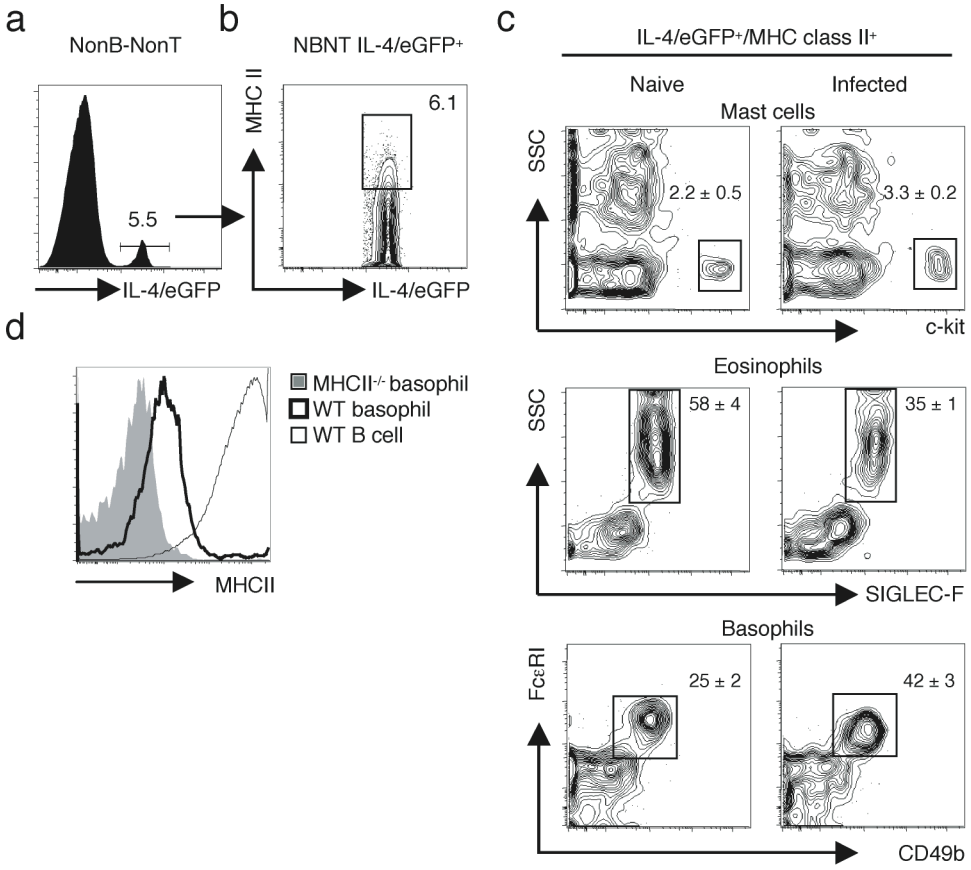

Figure 14. FcεRI^{+} CD49b^{+} basophils co-express MHC class II and IL-4/eGFP. Flow cytometry of splenocytes isolated from naive and *T. muris*-infected 4-get mice at day 14 post-infection. (a) IL-4/eGFP^{+} cells were identified from a non-B non-T (NBNT) cell gate (CD3^{-}B220^{-}CD19^{-}) and (b) analyzed for co-expression of MHC class II and IL-4/eGFP. (c) IL-4/eGFP^{+}, MHC class II^{+} NBNT cells were characterized for expression of c-Kit, siglec-F, FcεRI, and CD49b. Plots representative of *n* = 3 mice per group (a-c); numbers are mean +/- SEM (c). (d) MHC class II expression on WT basophils (heavy line), MHC class II$^{-/-}$ basophils (grey filled), or WT B cells (light line) isolated from naive mice. Plots representative of *n* = 3 mice per group.

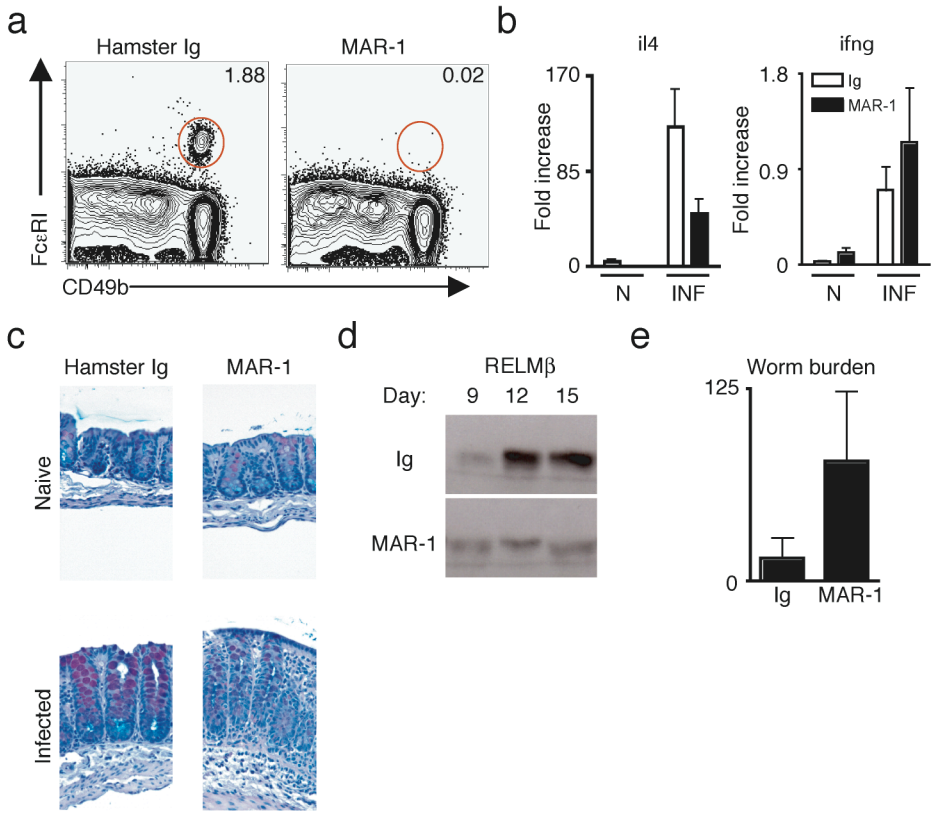

Figure 15. Depletion of FcεRI^{+} cells *in vivo* results in impaired immunity to *Trichuris* infection. (a) Flow cytometric analysis of splenic basophils from control Ig-treated or MAR-1-treated mice at day 21-post-infection. Plots gated on CD3^{-}B220^{-}CD19^{-} non-B non-T cells. (b) Real-time quantitative PCR of colon tissue from naive and infected Ig-treated or MAR-1-treated mice at day 21-post-infection; results represented as fold increase over naive Ig-treated controls. (c) Cecal sections from naive and infected Ig-treated or MAR-1-treated mice at day 21-post-infection stained for mucins with Alcian blue/periodic-acid Schiff. (d) Immunoblot of protein extracted from pooled fecal pellets of Ig-treated or MAR-1-treated mice at indicated days post-infection and

immunoblotted for RELMβ. (e) Cecal worm burdens at day 21 post-infection. Results are representative of two independent experiments of *n* = 3-4 mice per group (a-e).

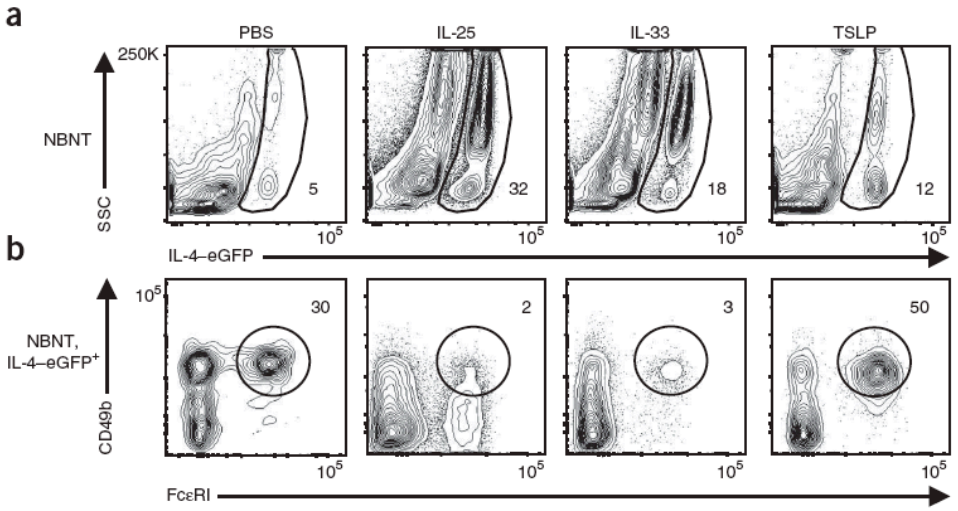

Figure 16. TSLP treatment selectively increases basophil frequencies *in vivo*. Flow cytometric analysis of basophil frequencies in the blood of mice treated daily for four days with rIL-25, rIL-33 or rTSLP. (a) Non-B non-T cells from the peripheral blood were analyzed for expression of IL-4/eGFP. Numbers indicate frequency of gated population. (b) Basophil frequencies in IL-4/eGFP$^+$ non-B non-T cell populations. Numbers indicate frequency of gated population. Results representative of at least two independent experiments with *n* = 3 mice per group.

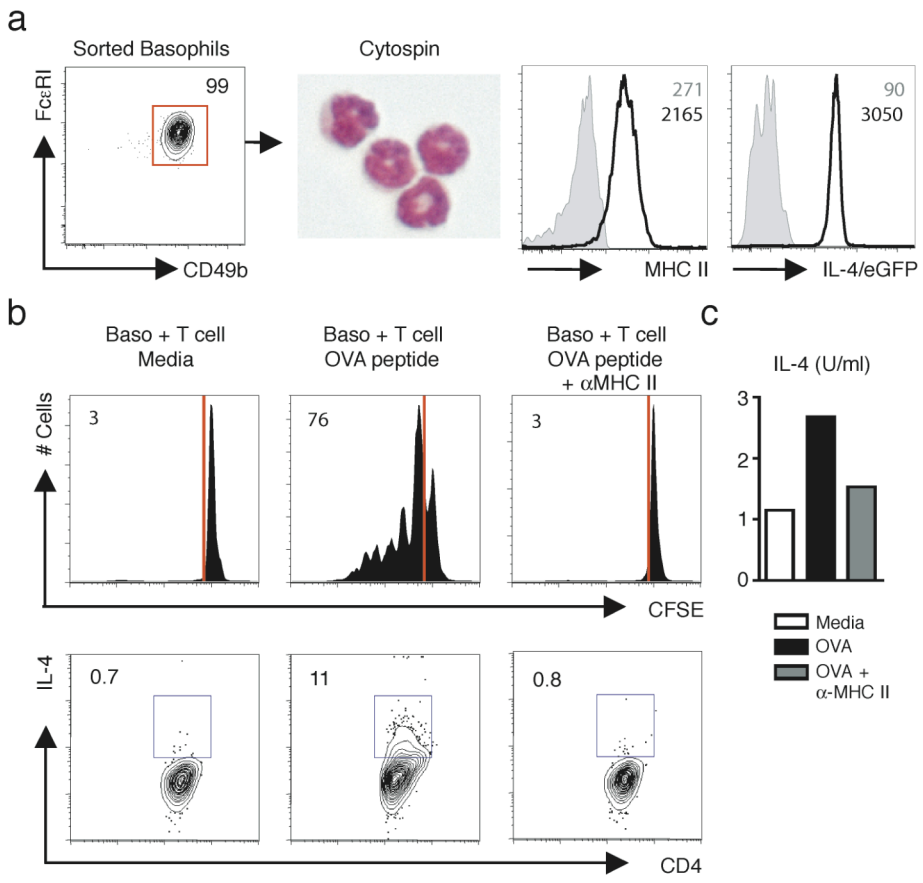

Figure 17. Basophils promote MHC class II-dependent antigen-specific CD4$^+$ T cell proliferation and Th2 cytokine production *in vitro*. (a) Sorted TSLP-elicited basophils were subjected to cytospin and stained with DiffQuick or examined by flow cytometry for expression of MHC class II and IL-4/eGFP (solid line) over fluorescence minus one (FMO) controls (shaded histrograms). Numbers in italics are mean fluorescence intensity. Plots are representative of four independent experiments with *n* = 5-10 mice pooled per experiment. (b) CFSE-dilution of DO11.10 CD4$^+$ T cells following 4 day co-culture with basophils in media, OVA peptide, or OVA peptide plus MHC class II blocking antibody (M5114) in upper panel. Lower panel indicates frequencies of IL-4$^+$ CD4$^+$ T cells by intracellular cytokine staining. (c) Supernatants from basophil-CD4$^+$ T

cell co-cultures in were analyzed for IL-4 secretion by ELISA. Results are representative of two independent experiments (b,c).

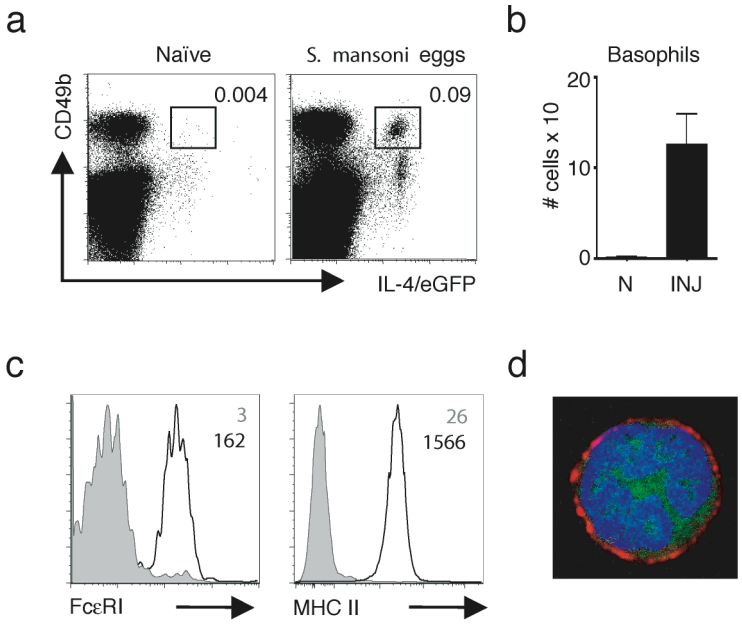

Figure 18. IL-4/eGFP^{+} MHC class II^{+} basophils are recruited to the draining LN following exposure to *Schistosoma mansoni* eggs. (a) Flow cytometry of basophil frequencies in popliteal LN from naive or *S. mansoni* egg-injected 4-get mice day 2 post-injection. (b) Total numbers of basophils in the lymph node of naive (N) or *S. mansoni* egg injected (INJ) mice. (c) Flow cytometry of sorted *S. mansoni*-elicited basophils stained for FcεRI or MHC class II. Grey histograms are expression on CD3^{+} cells, black lines are sorted basophils. Numbers indicate mean fluorescence intensity. (d) Confocal microscopy of sorted *S. mansoni*-elicited basophils. GFP (green); MHC class II (red); DAPI (blue). Results are representative of five independent experiments with *n* = 3-5 mice per group.

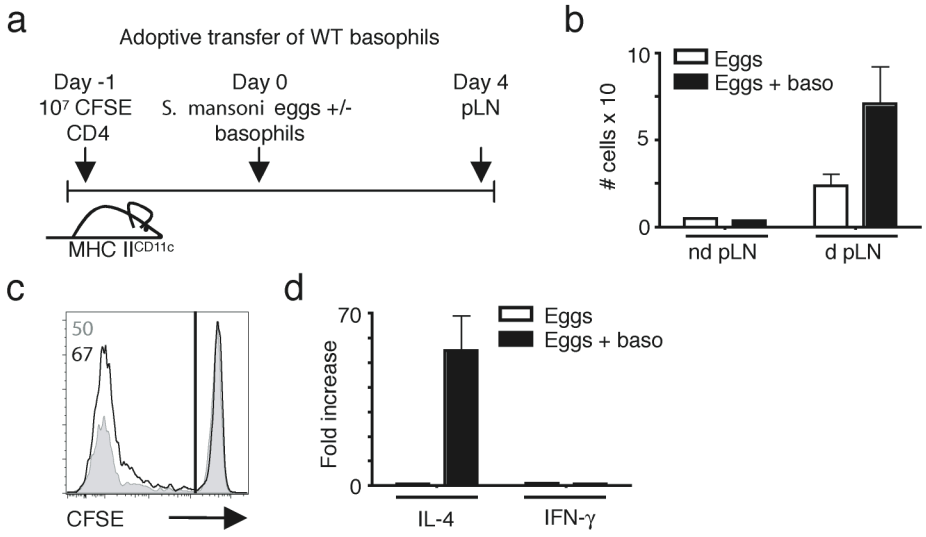

Figure 19. Basophils augment CD4$^+$ T cell proliferation *in vivo* following exposure to *Schistosoma mansoni* eggs. (a) Schematic of basophil transfer experiment. (b) Total numbers of CD4$^+$ cells in the non-draining (nd pLN) versus draining (d pLN) popliteal lymph nodes of MHC CII^{CD11c} that received either *S. mansoni* eggs alone or in combination with basophils. (c) Flow cytometry showing CFSE-dilution of donor CD4$^+$ T cells from MHC CII^{CD11c} that received either *S. mansoni* eggs alone (grey histogram) or with basophils (black line histogram). Numbers refer to percent of CFSE^{lo} cells. (d) IL-4 and IFN-γ mRNA expression in pLN from mice that received either *S. mansoni* eggs alone or with basophils; represented as fold-induction over eggs alone. Results represent *n* = 2 mice per group.

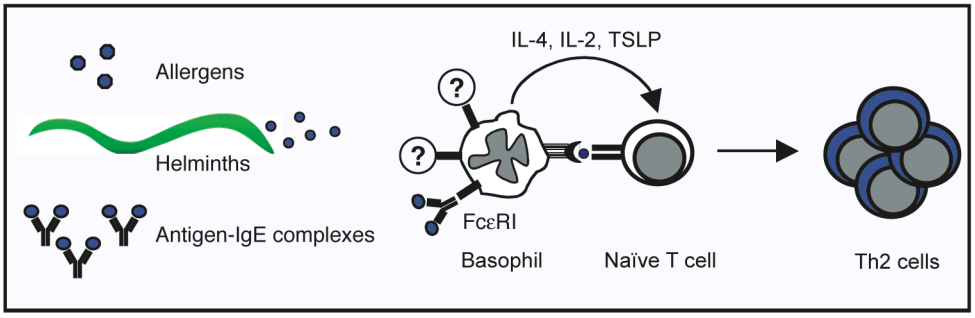

Figure 20. Basophils can act as antigen presenting cells to promote development of Th2 effector cell responses following exposure to protease allergens, helminth parasites and allergen-IgE complexes.

Chapter 4
Regulation of type 2 immunity and inflammation via IL-31-IL-31R interactions

4.1 Abstract

IL-31 is a helical cytokine made predominantly by $CD4^+$ T helper type 2 cells (Th2) originally identified as a pro-inflammatory cytokine in the skin. Here, we identify a novel function for endogenous IL-31- IL-31R interactions in limiting type 2 inflammation in the intestine and lung. In response to *Trichuris* infection, IL-31R$\alpha^{-/-}$ mice exhibited increased Th2 cytokine production in the mesenteric lymph nodes, developed enhanced goblet cell responses and accelerated worm expulsion compared to their WT counterparts. Additionally, in an acute model of lung inflammation, following intravenous injection of *Schistosoma mansoni* eggs, IL-31R$\alpha^{-/-}$ mice developed severe pulmonary inflammation, characterized by an increase in the area of granulomatous inflammation, increased numbers of RELM-α^+ cells, and enhanced collagen deposition compared to WT counterparts. *In vitro*, macrophages generated from IL-31R$\alpha^{-/-}$ mice promoted enhanced OVA-specific $CD4^+$ T cell proliferation and purified naïve $CD4^+$ T cells from IL-31R$\alpha^{-/-}$ mice exhibited enhanced proliferation and expression of Th2 cytokines, identifying a T cell- and macrophage-intrinsic regulatory function for IL-31R signaling. In contrast, the generation of $CD4^+$ Th1 cell-mediated IFN-γ responses *in vitro* and *in vivo* were normal in IL-31R$\alpha^{-/-}$ mice. Together, these data implicate IL-31R signaling as a novel negative regulatory pathway that specifically limits type 2 inflammation.

4.2 Introduction

The development, differentiation, and activation of immune cells are critically dependent upon the interaction of cytokine receptors with their cognate ligands. The largest of the cytokine receptor families is the type I receptor family, members of which can be categorized based upon their structure and use of common signaling receptor chains (Boulay *et al.*, 2003). Several conserved structural domains of type 1 cytokine receptors, including the signature WSXWS motif, have facilitated the in silico identification of novel family members. Using this approach the most recent addition to this family, the gp130-like monocyte receptor (GLM-R) or IL-31Rα, was identified (Ghilardi *et al.*, 2002). By sequence homology IL-31Rα exhibits strong similarity to gp130, a promiscuous type 1 receptor chain that serves as the signaling receptor for a number of ligands (Muller-Newen, 2003). However, as yet the only known functional ligand for IL-31Rα is IL-31 that signals through a heterodimeric complex composed of IL-31Rα and OSMRβ (Dreuw *et al.*, 2004; Diveu *et al.*, 2003; Ghilardi *et al.*, 2002). IL-31Rα expression has been detected on a wide range of cell types including macrophages, T cells, and epithelial cells (Dillon *et al.*, 2004b; Dreuw *et al.*, 2004; Diveu *et al.*, 2003; Ghilardi *et al.*, 2002) while IL-31 is produced by activated T cells, predominantly $CD4^+$ T helper type 2 (Th2) cells (Dillon *et al.*, 2004b). *In vitro* studies showed that signaling downstream of the IL-31R results in the phosphorylation of STAT1, STAT3, and STAT5 as well as activation of the p38 MAPK, ERK1,2, and JNK1,2 signaling pathways (Chattopadhyay *et al.*, 2007; Ip *et al.*, 2007; Dillon *et al.*, 2004b; Dreuw *et al.*, 2004; Ghilardi *et al.*, 2002). In epithelial cells IL-31 treatment has been linked to increased production of a broad range of cytokines and chemokines including IL-6, IL-8, EGF, VEGF, MCP-1, and GRO1α and can result in either the promotion or inhibition of cell

proliferation dependent upon dose and target cell type (Chattopadhyay *et al.*, 2007; Ip *et al.*, 2007; Dillon *et al.*, 2004b). Structurally similar to the helix bundle cytokine IL-6, IL-31 belongs to the IL-6/IL-12 family of cytokines that includes IL-6, IL-11, IL-12, IL-23, IL-27, cardiotrophin-1 (CT-1), cardiotrophin like cytokine (CLC), leukemia inhibitory factor (LIF), and oncostatin M (OSM) (Boulay *et al.*, 2003), many of which have important pro- and anti-inflammatory functions as well as roles in regulating the development and differentiation of immune cells (Hunter, 2005; Hofmann *et al.*, 2002). Recent research has highlighted the critical roles that members of this cytokine family play in the regulation of immunity and inflammation and underscore the potential for manipulating these signaling pathways for therapeutic benefit in a wide range of inflammatory diseases (Batten and Ghilardi, 2007; Hunter, 2005; Nishimoto and Kishimoto, 2004).

While *in vitro* assays have begun to define the signaling pathways involved downstream of IL-31-IL-31R interactions, relatively little is known about the functional consequences of these interactions *in vivo*. Transgenic mice that express IL-31 from a ubiquitous or T cell-specific promoter develop spontaneous skin inflammation characterized by thickening of the epidermis, alopecia, and pruritis (Dillon *et al.*, 2004b). That study, combined with subsequent analyses in humans correlating IL-31 expression and dermatitis, suggested a role for IL-31-IL-31R interactions in regulating inflammation in the skin (Bilsborough *et al.*, 2006; Neis *et al.*, 2006; Sonkoly *et al.*, 2006; Takaoka *et al.*, 2006; Takaoka *et al.*, 2005; Dillon *et al.*, 2004b). However, the functional significance of endogenous IL-31-IL-31R interactions in influencing innate and adaptive immune responses remains unknown. In this chapter, we describe the generation of IL-31R$\alpha^{-/-}$ mice, identify a role for IL-31R signaling in limiting the magnitude of type 2 inflammation

in the intestine and the lung and demonstrate effects of IL-31R deletion on both innate and adaptive immune cells.

4.3 Methods

4.3.1 Mice

Wild type C57BL/6 mice and OTII transgenic mice were ordered from Jackson Laboratories. All mice were bred in a specific-pathogen free environment at the University of Pennsylvania. All experiments were performed following the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. IL-31R$\alpha^{-/-}$ mice were generated by constructing a targeting vector that deleted the cytokine receptor homology (CRH) domain of IL-31Rα (encoded by exons 5-8 of the murine transcript) from the following DNA fragments: a 5' homology arm of 5289 base pairs was PCR-amplified fragment from C57BL/6 genomic DNA, using primer 5'-TTAATTAAATCTACATGTGTGCGGAGGC-3' and primer 5'-GCGGCCGCATGTTCTCTGGCTTAGTCGGCAGG-3'; a PGK-neo resistance cassette; a 3' homology arm of 1108 bp short arm defined by primers 5'-GGCGCGCCAGGGGTGGTGGTGGATGGAT-3' and primer 5'-GGCGCGCCGTCTACAGGGTTAACCTA-3' (Fig 1A), and an HSV driven thymidine kinase selection cassette for negative selection. This construct was electroporated into C57BL/6 embryonic stem (ES) cells, and homologous recombination occurred in 4 out of 200 clones following selection with G418 and Gancyclovir. To verify correct targeting of the locus, genomic DNA from ES cells and animals was analyzed by southern blot. Digestion with SacI followed by hybridization of membranes with probe 1 (a 2400 bp

genomic DNA fragment obtained by PCR with oligos 5'-GAGCTCCCGGGATCACGTCC-3' (sense) and 5'-AGGCCTCCTCTGGAGCTGGG-3' (antisense) yielded a 11904 bp fragment for the wild-type allele and an 8877 bp fragment for the correctly targeted mutant allele. Similarly, digestion of genomic DNA with BamHI followed by hybridization of membranes with probe 2 (a 1028 bp genomic DNA fragment obtained by PCR with oligos 5'-ATCGATAGGTTCAGTGGTA-3' (sense) and 5'-AAGTACTGTATGTGGTAGCC-3' (antisense) yielded a 6990 bp fragment for the wild-type allele and an 9401 bp fragment for the correctly targeted mutant allele. Two ES cell clones were injected into blastocysts, and animals that transmitted the mutant allele in their germline were obtained. For genotyping, a PCR-based method with a common antisense primer (5'-GGCAGTAACTGAAGTAACAG-3') in combination with wild-type- (5'- CAAAGTCACAATGTAGCTGG-3') and knockout-specific (5'-CGCCTTCTTGACGAGTTCTT-3') sense primers was used. This primer-triplet amplifies a 492 bp fragment for the wild-type allele and an 808 bp fragment for the mutant allele. PCR was carried out in a PE9700 thermocycler (Perkin Elmer) using the following conditions: 1 cycle of 94º C, 4'; 35 cycles of 94º C, 30", 60º C, 30", 72º C, 60"; 1 cycle of 72º C, 7'. IL-31R$\alpha^{-/-}$ mice were born at Mendelian frequencies and were equivalent in size, weight, and fertility to littermate controls. No abnormalities in organ development or inflammation were found upon histopathological examination, and analysis of clinical chemistry and hematology parameters did not reveal differences between WT and IL-31R$\alpha^{-/-}$ mice.

4.3.2 FACs analysis of blood cell subsets

Spleens, thymi, and lymph nodes were isolated from 6-8 week old mice and single cell suspensions prepared. Peripheral blood was obtained by cardiac puncture and treated

with EDTA to prevent coagulation, and erythrocytes were lysed using ACK lysing buffer (Biosource). All cells were incubated for 30 minutes on ice in Hanks balanced salt solution supplemented with 2 % heat inactivated bovine calf serum. Cells were stained with fluorochrome-conjugated antibodies against CD4, CD8, CD11b, CD11c, CD25, CD62L, B220, F4/80, and NK1.1 and analyzed using a FACSCalibur flow cytometry system (BD Biosciences).

4.3.3 Parasites

Isolation of *Trichuris* eggs was performed as described previously (Artis *et al.*, 2004c). Mice were infected orally with 200 embryonated eggs and parasite burdens in the ceca determined microscopically at indicated days after infection. Schistosoma mansoni eggs were isolated as previously described. Mice were injected with 5000 *S. mansoni* eggs intravenously and animals sacrificed at day 14 post-injection. *Leishmania major* parasites (MHOM/IL/80/Friedlin) were grown in Grace's insect culture medium (Life Technologies) supplemented with 20% heat-inactivated FBS (HyClone Laboratories), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich). Soluble *Leishmanial* Ag (SLA) was prepared as previously described (Scott *et al.*, 1987). Mice were injected in the hind left footpad with 5 x 10^6 parasites. Footpad swelling was measured weekly using digital calipers (Mitutoyo), and lesion size was determined by subtracting the size of the uninfected contralateral footpad from the size of the infected footpad. To quantify parasites in the footpad, single-cell suspensions of lesions were prepared and plated in two-fold serial dilutions (initial dilution of 1:100) in Grace's insect culture medium. Each sample was plated in quadruplicate, and the mean of the negative log parasite titer was determined after 7 days of culture at 26°C.

4.3.4 Histology and immunofluorescence

Cecal tips from naïve or *Trichuris*-infected mice were removed, rinsed with PBS and fixed in 4% paraformaldehyde-fixed followed by paraffin embedding. 5μm cecal tissue sections were stained for mucins with Periodic Schiffs/Alcian blue and stained by immunofluorescence for RELMβ or Gob5 as described previously (Artis *et al.*, 2004b). Lungs from naïve or *S. mansoni* egg-injected mice were inflated with 4% PFA, embedded in paraffin, cut in 5μm sections, and stained with H&E or Masson's trichrome to indicate collagen deposition. H&E sections were analyzed using Openlab software to assess the area of egg-induced inflammation by subtracting the area of the egg from the total area of the granuloma for each granuloma in a section. Paraffin sections were analyzed for expression of RELM-α by immunofluorescence via incubation with an anti-RELM-α antibody (1:1000, Peprotech) followed by addition of a Cy3-conjugated anti-rat secondary antibody (1:600, Jackson Laboratories).

4.3.5 ELISAs

Single cell suspensions of draining mesenteric lymph nodes from naïve or infected mice and plated at 5 million per ml in complete media (DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol) and stimulated for 48 hours with either media alone or 1μg/ml of anti-CD3/CD28 (eBioscience). Single cell suspensions of draining cranial mediastinal lymph nodes from *S. mansoni* egg-injected mice or peripheral lymph nodes from naïve mice were plated at 1.5 million per ml in complete media and stimulated for 48 hours with either media alone or 20μg/ml of soluble *S. mansoni* egg antigen (SEA). Supernatants were assayed for IL-4, IL-5, IL-13 and IFN-γ using standard sandwich ELISA protocols (eBioscience). Serum was isolated

by cardiac puncture and assayed by ELISA for IgE using OptIEA IgE kit (PharMingen). For antigen-specific IgG1 ELISAs, plates were coated with 5 μg per ml of *Trichuris* antigen prepared as previously described (Bancroft *et al.*, 1998); secondary anti-mouse IgG1 HRP antibody was purchased from BD Pharmingen.

4.3.6 Western blot

Western blot analysis was performed as described previously (Artis *et al.*, 2004b). Briefly, 30 μg of protein was isolated from pooled fecal pellets collected at various time points post-infection and resolved by SDS-PAGE followed by immunoblotting for RELMβ with a polyclonal rabbit α-murine RELMβ antibody (Peprotech).

4.3.7 T cell proliferation assays

Naïve $CD4^+$ T cells were isolated via negative depletion with a naïve $CD4^+$ T cell column from RnD Systems (purity >90% $CD4^+$, $CD44^{low}$, $CD62L^{high}$), labeled with CFSE (5μg/ml, Molecular Probes) and stimulated with plate-bound anti-CD3/anti-CD28 (1ug/ml, eBioscience) under neutral or T_H2 [IL-4 (40ng/ml, RnD Systems), anti-IL-12 (10μg/ml, C17.8), and anti-IFN-γ (50μg/ml, XMG-6)] polarizing conditions. At day 4 cells were stimulated with PMA (50ng/ml Sigma), ionomycin (500ng/ml Sigma), and Brefeldin A (10μg/ml Sigma) for 4 hours. Following stimulation cells were surface stained for CD4, fixed, acquired on a FACSCalibur (BD Biosciences) cytometer and analyzed using FloJo software (Tree Star) for proliferation. Purified $CD4^+$ T cells for in vitro T_H1 cell differentiation assays were obtained by subjecting whole spleen and lymph node suspensions to negative selection via incubation with hybridoma supernatants (αB220, αF_CR, αCD8, αMHCII) followed by magnetic bead purification (QIAGEN), CFSE-labeled and stimulated with anti-CD3/anti-CD28 (1μg/ml eBioscience) in the presence of rIL-12

(1ng/ml) and anti-IL-4 (11B11 10μg/ml). On day four, cells were harvested and subjected to flow cytometry as described above.

4.3.8 Macrophage: T cell co-cultures

Macrophages were derived in vitro as follows. Bone marrow from C57BL/6 or IL-31Rα$^{-/-}$ mice was plated on 10cm Petri dishes in DMEM supplemented with 100 μg/ml penicillin/streptomycin, 10% FCS, 1% sodium pyruvate, 25mM HEPES, and 30% L-cell-conditioned media. Media was changed at 3 days and cells were harvested at day seven. Bone marrow-derived macrophages from WT or IL-31Rα$^{-/-}$ mice were plated at 5x10^{5}cells/well and pulsed overnight with 500μg/ml OVA protein (Worthington) in the presence or absence of IL-4 (40ng/ml, RnD Systems). Purified OTII T cells were obtained by passing whole spleen and lymph node suspensions over T cell enrichment columns (R&D Systems). OTII T cells were CFSE-labeled and co-cultured with macrophages at a 4:1 ratio at which time anti-IFN-γ and IL-4 were added for T_H2-polarizing conditions in the IL-4-primed macrophage cultures. At day 4 cells were stimulated with PMA, ionomycin, and Brefeldin A as described above for 4 hours and analyzed by flow cytometry. For T_H1-polarizing conditions, macrophages were primed with 10ng/ml LPS and OVA as above. OT II T cells were CFSE-labeled and added at a 4:1 ratio with IL-12 (1ng/ml) and anti-IL-4 (11B11 10μg/ml).

4.3.9 RNA isolation and real-time PCR

RNA was purified from cells in culture per instructions using an RNeasy Kit (QIAGEN). cDNA was generated per standard protocol with Superscript reverse transcriptase (Invitrogen) and used as input for real-time PCR. Taqman® PCR reactions were run at standard conditions using an ABI 7500 sequence detector. Real-time data was analyzed

using the ΔΔCT method whereby actin served as the endogenous gene. Taqman® Assays on Demand (Applied Biosystems) were used to measure β-actin, IL-4, IL-13, and IFN-γ message levels.

4.4 Results

4.4.1 Generation and characterization of IL-31Rα$^{-/-}$ mice

To determine the functions of endogenous IL-31-IL-31R interactions, IL-31Rα$^{-/-}$ mice were generated by homologous recombination. The four exons encoding the ligand-binding cytokine receptor homology domain (exons 5-8) were replaced by a neomycin resistance cassette (**Fig. 21a**). Correct homologous recombination was confirmed by Southern blot analysis (**Fig. 21b,c**) and the absence of IL-31Rα expression confirmed by Taqman PCR (**Fig. 21d**). Comprehensive flow cytometric analysis of thymocytes, splenocytes and lymph node cells did not indicate any significant differences between naïve WT and IL-31Rα$^{-/-}$ animals in the composition of immune cells in these compartments (**Table I**). Importantly, analysis of peripheral blood did not reveal significant anemia or thrombocytopenia (data not shown), which has been reported for mice deficient in OSMRβ (Tanaka *et al.*, 2003).

4.4.2 IL-31Rα$^{-/-}$ mice exhibit enhanced Th2 cytokine and antibody responses following *Trichuris* infection

The over-expression of IL-31 in a transgenic murine model was associated with skin inflammation with characteristics similar to human atopic dermatitis, suggesting a pro-inflammatory role for IL-31R signaling (Bilsborough *et al.*, 2006; Neis *et al.*, 2006; Sonkoly *et al.*, 2006; Takaoka *et al.*, 2006; Takaoka *et al.*, 2005; Dillon *et al.*, 2004b).

However, the functional significance of endogenous IL-31-IL-31R interactions in regulating immunity and inflammation is currently unknown. Given the predominant expression of IL-31 in $CD4^+$ Th2 cells and its association with type 2 inflammation in the skin, we investigated the potential regulatory functions of IL-31-IL-31R interactions in an *in vivo* model of Th2 cytokine-dependent immunity and inflammation in the intestine. WT and IL-31R$\alpha^{-/-}$ mice were infected with the gastrointestinal helminth *Trichuris muris*. As described previously in chapters 2 and 3, immunity to *Trichuris* is critically dependent upon the generation of $CD4^+$ Th2 cells. At day 18 post-infection, mLN cells were isolated and polyclonally stimulated with anti-CD3 and anti-CD28 for 48 hours to assess cytokine production. Consistent with previous studies (Owyang *et al.*, 2006; Artis *et al.*, 2004c; Artis *et al.*, 2002; Bancroft *et al.*, 1998), mLN cells isolated from infected WT mice produced detectable IL-4, IL-5, and IFN-γ following polyclonal stimulation (**Fig. 22a**). However, mLN cells isolated from infected IL-31R$\alpha^{-/-}$ mice secreted significantly higher levels of IL-4, elevated levels of IL-5 and significantly lower amounts of IFN-γ compared to that observed in infected WT mLN cells (**Fig. 22a**).

The generation of a protective Th2 cytokine response to *Trichuris* results in IL-4-dependent class-switching and B cell production of IgE and IgG1 antibodies (Bancroft *et al.*, 1998). To further assess the magnitude of the Th2 cytokine-dependent responses in both WT and IL-31R$\alpha^{-/-}$ mice, serum Ig levels were determined by ELISA. At day 18 post-infection, the amount of serum IgE detected in WT mice increased approximately 2-fold over that of naïve WT mice. In contrast, serum IgE levels from infected IL-31R$\alpha^{-/-}$ mice increased 4-fold over naïve IL-31R$\alpha^{-/-}$ mice and almost 10-fold over naïve WT mice (**Fig. 22b**). In addition to increased total IgE production, antigen-specific IgG1

antibodies were significantly increased in the serum of day 18 infected IL-31Rα$^{-/-}$ mice compared to infected WT mice (**Fig. 22c**), consistent with the observed increases in Th2 cytokine production in the mLN cells from infected IL-31Rα$^{-/-}$ mice (**Fig. 22a**).

4.4.3 Enhanced goblet cell responses and accelerated expulsion of *Trichuris* in IL-31Rα$^{-/-}$ mice

Trichuris infection is associated with Th2 cytokine-mediated changes in intestinal epithelial cell proliferation and differentiation, including increased turnover and goblet cell differentiation, that contribute to worm expulsion (Artis and Grencis, 2008; Zaph *et al.*, 2007; Nair *et al.*, 2006; Cliffe *et al.*, 2005). To determine whether the loss of IL-31R signaling influenced these aspects of type 2 inflammation in the intestine, WT and IL-31Rα$^{-/-}$ mice were infected with *Trichuris* and cecal sections stained with periodic Schiff's/Alcian blue to assay goblet cell hyperplasia and mucin production. While both WT and IL-31Rα$^{-/-}$ mice exhibited an infection-induced increase in goblet cell numbers and mucin production (**Fig. 23a**), goblet cell hyperplasia was markedly enhanced in IL-31Rα$^{-/-}$ mice with significantly increased numbers of goblet cells per cecal crypt (**Fig. 23b**). In previously published studies, microarray analysis comparing ceca from naïve and infected mice identified the goblet cell-specific resistin-like molecule, RELMβ, as a highly differentially expressed gene in the intestine following *Trichuris* infection (Artis *et al.*, 2004b). Immunofluoresence staining of cecal sections from infected WT and IL-31Rα$^{-/-}$ mice at day 18 post-infection indicated the dominant cellular source of RELMβ in both WT and IL-31Rα$^{-/-}$ mice was goblet cells and consistent with increased goblet cell numbers, RELMβ expression was elevated in IL-31Rα$^{-/-}$ mice (**Fig. 23c**). Expression of Gob5 (mCLCA3), another goblet cell-specific gene regulated by Th2 cytokines and

associated with type 2 inflammation, was also enhanced in infected IL-31Rα$^{-/-}$ mice compared to infected WT mice (**Fig. 23d**). Maximal RELMβ secretion is correlated with worm expulsion and because its expression is STAT-6-responsive, the measurement of secreted RELMβ in fecal pellets can be an indicator of the kinetics of a developing Th2 cytokine response in infected mice. WT and IL-31Rα$^{-/-}$ mice were infected with *Trichuris* and secreted RELMβ measured by Western blot over the course of infection. As expected, RELMβ secretion was minimal in naïve WT mice, induced by day 14, and was maximal between days 14 and 16 post-infection (**Fig. 23e**). In contrast to their WT counterparts, naïve IL-31Rα$^{-/-}$ mice constitutively secreted more RELMβ and following infection, the magnitude of RELMβ secretion was substantially enhanced (**Fig. 23e**). Previous reports have identified direct effects of IL-31R signaling on epithelial cells including changes in proliferation and expression of various chemokines and cytokines. Thus, the changes in goblet cell proliferation following *Trichuris* infection could be a direct effect of IL-31-IL-31R signaling on intestinal goblet cell responses or an indirect consequence of elevated expression of Th2 cytokines. Notwithstanding that, these data show that in the absence of IL-31R signaling there is enhanced intestinal type 2 inflammation associated with significantly increased goblet cell hyperplasia and increased expression of the goblet cell-associated genes RELMβ and Gob5.

Given that IL-31Rα$^{-/-}$ mice exhibited enhanced Th2 cytokine responses following *Trichuris* infection, we sought to determine whether IL-31Rα$^{-/-}$ mice would more efficiently clear infection than their WT counterparts. To test this, WT and IL-31Rα$^{-/-}$ mice were infected with 200 embryonated *Trichuris* eggs and sacrificed at days 12, 21,

and 34 post-infection. There was no significant difference in worm establishment at day 12 (WT 163+/-58; IL-31R$\alpha^{-/-}$ 125+/-32). Expulsion of *Trichuris* in WT C57BL/6 mice occurs between days 18-21 post-infection and as expected, at day 21 post-infection WT mice had begun to expel *Trichuris* with an average cecal worm burden below 40 (**Fig. 24**). However, IL-31R$\alpha^{-/-}$ mice exhibited a nearly 10-fold decrease in worm burden compared to their WT counterparts at the same time point (**Fig. 24**). Collectively, these data suggest that in contrast to the promotion of skin inflammation observed with IL-31 over-expression, endogenous IL-31R interactions negatively regulative the parasite-induced Th2 cytokine responses that influence intestinal immunity and inflammation.

4.4.4 Mice deficient in IL-31Rα develop exacerbated type 2 inflammation in the lung following Schistosoma mansoni egg injection

The apparent difference in the biological function of IL-31R signaling in the skin versus the intestine led to the hypothesis that IL-31-IL-31R interactions may regulate Th2 cytokine-dependent inflammation differentially depending on the tissue site. To test the function of IL-31-IL-31R interactions in the lung, we utilized a model of acute lung inflammation induced by intravenous injection of *Schistosoma mansoni* eggs. Upon i.v. injection, *S. mansoni* eggs travel through the bloodstream and become lodged in the small blood vessels of the lung where they induce the formation of IL-4- and IL-13-dependent type 2 granulomas (Wynn and Cheever, 1995). Following delivery of *S. mansoni* eggs, WT mice developed characteristic egg-induced granulomas composed of a cuff of epithelioid macrophages surrounded by granulocytes and lymphocytes (**Fig. 25a**). In contrast, egg-injected IL-31R$\alpha^{-/-}$ mice developed more severe inflammation in the lung parenchyma associated with increased granulocyte infiltration (**Fig. 25a**) and a greater than two-fold increase in the average area of inflammation surrounding each egg

(**Fig. 25b**). Draining mediastinal lymph node (LN) cells were isolated from egg-injected WT and IL-31R$\alpha^{-/-}$ mice and re-stimulated with *S. mansoni* egg antigen (SEA). The *S. mansoni* egg-induced granuloma model can either employ pre-immunization with *S. mansoni* eggs systemically, followed by i.v. challenge or direct i.v. primary challenge without prior sensitization. Direct primary challenge i.v. results in relatively low expression of Th2 cytokines but the development of granulomas in the lung parenchyma (Wynn *et al.*, 1994). Consistent with this, following primary challenge with *S. mansoni* eggs, LN cells isolated from injected WT mice produced detectable levels of IL-5 but no IL-4 or IL-13. In contrast, LN cells from IL-31R$\alpha^{-/-}$ mice exhibited significantly higher levels of IL-4, IL-5, and IL-13 following antigen-specific re-stimulation (**Fig. 25c**). No cytokines were detected in SEA-stimulated lymph node cells from naïve WT or IL-31R$\alpha^{-/-}$ mice (data not shown). *S. mansoni* egg-injected IL-31R$\alpha^{-/-}$ mice also exhibited significantly increased serum IgE levels compared to WT mice (**Fig. 25d**), consistent with increased expression of Th2 cytokines.

Alternatively-activated macrophages (AAMs) are a dominant feature of *S. mansoni* egg-induced granulomas(Herbert *et al.*, 2004) and differentiation of AAMs is dependent upon Th2 cytokines. These macrophages possess a unique molecular signature, including expression of arginase and RELM-α, and have been implicated in granuloma formation, tissue remodeling, and fibrosis (Gordon, 2003; Nair *et al.*, 2003; Raes *et al.*, 2002). Consistent with exacerbated expression of Th2 cytokines and increases in AAMs, the frequency of RELM-α^{+} cells was increased in the lung granulomas of egg-injected IL-31R$\alpha^{-/-}$ mice (**Fig. 25e**). RELM-α and arginase expression have been correlated with

increases in collagen deposition and fibrosis (Liu *et al.*, 2004; Wynn, 2004; Sandler *et al.*, 2003) and accordingly, elevated collagen deposition was observed in the lungs of injected IL-31R$\alpha^{-/-}$ mice compared to WT counterparts (**Fig. 25f**). In total, the increased granuloma sizes, exacerbated Th2 cytokine production, high serum IgE levels, elevated frequency of RELM-α^{+} cells, and enhanced collagen deposition in the lung parenchyma are indicative of exaggerated acute type 2 inflammation in the absence of IL-31R signaling.

4.4.5 WT and IL-31R$\alpha^{-/-}$ B cells exhibit equivalent proliferation and survival *in vitro*.

The significant increases in IgE following *S. mansoni* egg injection and *Trichuris*-specific IgG1 antibodies suggest that IL-31-IL-31R interactions could have a direct effect on B cell function. Supporting this hypothesis, overexpression of IL-31 resulted in aberrant B cell to T cell ratios in the peripheral lymph nodes of IL-31 transgenic mice. However no analysis of direct effects of IL-31-IL-31R signaling on B cells were performed (Dillon *et al.*, 2004b). To first determine whether B cells may be responsive to IL-31, RNA was isolated from naïve and CpG-stimulated CD23^{+} B cells and IL-31Rα expression analyzed by real-time quantitative PCR. Expression of IL-31Rα in naïve B cells was similar to that observed in naïve CD4^{+} T cells while IL-31 expression was undetectable (data not shown). To determine whether there are inherent differences in the numbers or percentages of B cell subsets between WT and IL-31R$\alpha^{-/-}$ mice, splenocytes were isolated and stained with antibodies against B cell surface markers CD21/35 (complement component receptors) and CD23 (low affinity IgE receptor) and analyzed by flow cytometry. No differences were observed in numbers (data not shown) or percentages of marginal zone (CD21/35^{+}CD23^{int}) or follicular (CD21/35^{-}CD23^{high}) B cells

between WT and IL-31Rα$^{-/-}$ mice (**Fig. 26a**), indicating that IL-31R signaling does not influence the composition of the mature B cell compartment. To determine whether peripheral B cell responses were influenced by IL-31R signaling, purified WT and IL-31Rα$^{-/-}$ splenic B cells were treated *in vitro* with the TLR9 agonist CpG, a B cell mitogen, in the presence or absence of recombinant IL-31. After four days in culture their proliferative capacity and survival were determined by CFSE dilution and TOPRO staining respectively. Flow cytometric analysis revealed no significant differences in either proliferation or survival between CpG-stimulated WT and IL-31Rα$^{-/-}$ B cells (**Fig. 26b**). Similar results were observed when B cells were stimulated with anti-B cell receptor antibodies (data not shown). Together, these data suggest that there is no intrinsic role for IL-31Rα in influencing B cell development, proliferation, or survival and that exaggerated antibody responses in *Trichuris*-infected IL-31Rα$^{-/-}$ mice may be an indirect effect of enhanced CD4^{+} Th2 cell responses rather than a direct effect on B cells.

4.4.6 IL-31R signaling influences the proliferation and cytokine expression of naïve CD4^{+} T cells following polyclonal stimulation

Previous studies have reported that both IL-31 and IL-31R are upregulated by activated human T cells (Dillon *et al.*, 2004b). To test whether IL-31R signaling directly influenced CD4^{+} T cell function and could contribute to the dysregulated type 2 inflammation in the intestine and lung we observed following *Trichuris* infection and *S. mansoni* egg injection of IL-31Rα$^{-/-}$ mice, purified naïve CD44^{low}CD62L^{high} CD4^{+} T cells from WT or IL-31Rα$^{-/-}$ mice were CFSE-labeled and stimulated under neutral conditions with plate-bound anti-CD3/anti-CD28. Four days following stimulation IL-31Rα$^{-/-}$ CD4^{+} T cells exhibited

enhanced proliferation compared to WT counterparts (**Fig. 27a**). Upon activation, purified naïve $CD4^+$ T cells from IL-31R$\alpha^{-/-}$ mice also expressed significantly higher levels of IL-4 mRNA and consistently elevated levels IL-13 mRNA (**Fig. 27b**). However, under Th2-polarizing conditions, similar proliferation and expression of IL-4 and IL-13 was observed between WT and IL-31R$\alpha^{-/-}$ $CD4^+$ T cells suggesting that IL-31-IL-31R interactions do not regulate differentiated Th2 cells (data not shown). Rather, these data demonstrate that IL-31R signaling can directly influence the proliferation and expression of Th2 effector cytokines by naïve $CD4^+$ T cells.

4.4.7 IL-31R$\alpha^{-/-}$ macrophages exhibit enhanced accessory cell function

As discussed above, macrophage infiltration is a characteristic of *S. mansoni* egg-induced granulomas and the alternative activation of macrophages is necessary for survival of murine hosts during *S. mansoni* infection (Herbert *et al.*, 2004). It has been previously reported that resting and activated human monocytes express IL-31Rα (Dillon *et al.*, 2004b; Diveu *et al.*, 2003; Ghilardi *et al.*, 2002) and we have observed the induction of IL-31Rα expression on stimulated murine macrophages (data not shown). To address a role for IL-31R signaling in the regulation of macrophage function WT and IL-31R$\alpha^{-/-}$ macrophages were pulsed overnight with OVA protein in the presence or absence of recombinant IL-4 and co-cultured with CFSE-labeled OTII transgenic OVA-specific T cells. Flow cytometric analysis of WT and IL-31R$\alpha^{-/-}$ bone marrow-derived macrophages revealed no significant differences in surface expression of MHC class II, CD80, or CD40 (**Fig. 28a**). However, under both neutral and Th2-polarizing conditions, T cells co-cultured with IL-31R$\alpha^{-/-}$ macrophages proliferated to a greater extent than those cultured with WT macrophages (**Fig. 28b**). When macrophages were stimulated

with LPS and co-cultured with OVA-specific $CD4^+$ T cells under Th1-polarizing conditions there were no differences in $CD4^+$ T cell proliferation (data not shown). These data support a role for IL-31R signaling in limiting innate responses by negatively regulating the T-cell stimulatory function of macrophages.

4.4.8 IL-31R signaling specifically limits type 2 immune responses

While IL-31 is made predominantly by $CD4^+$ Th2 cells, IL-31 mRNA expression is also induced by $CD4^+$ T cells stimulated under both neutral and Th1 conditions (Dillon *et al.*, 2004b). We therefore investigated whether IL-31-IL-31R signaling could negatively regulate type 1 immune responses *in vitro* or *in vivo*. Purified $CD4^+$ T cells from naïve WT and IL-31R$\alpha^{-/-}$ mice stimulated polyclonally under Th1 polarizing conditions exhibited no differences in their ability to proliferate or acquire expression of IFN-γ as measured by intracellular cytokine staining (**Fig. 29a**), real-time PCR (**Fig. 29b**), or ELISA (**Fig. 29c**) indicating that IL-31R signaling does not influence Th1 cell differentiation *in vitro*. Furthermore, following infection with the intracellular parasite *Leishmania major,* immunity to which is critically dependent upon IFN-γ production by $CD4^+$ T cells (Sacks and Noben-Trauth, 2002), WT and IL-31R$\alpha^{-/-}$ mice exhibited equivalent footpad swelling (**Fig. 29d**), with peak lesions observed at four weeks post-infection and control of parasite replication (**Fig. 29e**) and resolution of cutaneous inflammation occurring by week twelve. Consistent with the *in vitro* T cell differentiation data, the frequency of *Leishmania*-specific IFN-γ producing $CD4^+$ T cells in the draining popliteal lymph node was similar in infected WT and IL-31R$\alpha^{-/-}$ mice, indicating normal Th1 cell polarization in IL-31R$\alpha^{-/-}$ mice (**Fig. 29f**). IL-31R$\alpha^{-/-}$ mice also displayed equivalent type 1 cytokine-dependent resistance following infection with *Listeria monocytogenes* (Nico Ghilardi,

personal communication). These results suggest that IFN-γ responses are not influenced by IL-31R signaling and support a role for IL-31-IL-31R interactions as a distinct negative feedback pathway operating during type 2 inflammation.

4.5 **Discussion**

We have identified IL-31-IL-31R signaling as a novel negative regulator of type 2 inflammation in the lung and intestine that can directly influence the function of antigen-presenting cells and $CD4^+$ T cells. Previous studies have proposed a pro-inflammatory role for IL-31 in the skin as IL-31 transgenic mice develop a skin pathology resembling atopic dermatitis (Dillon *et al.*, 2004b). Although there was no elevation in IgE (characteristic of atopic dermatitis) or assessment of Th2 cytokines, these studies suggested that IL-31 could promote type 2 inflammation (Dillon *et al.*, 2004b). There are a number of explanations for the apparent differences in the putative positive effects of IL-31 in the skin versus the lung and intestine where IL-31 has a negative effect on Th2 cytokine production. One of the key differences between our studies and the work with IL-31 transgenic mice is the deletion of the endogenous receptor versus over-expression of the ligand. The expression of IL-31 at levels that are super-physiological could explain these disparate outcomes. Additionally, as with other members of the type 1 cytokine receptor family, deciphering the functional biology of IL-31 and IL-31Rα may be complicated by promiscuous cytokine and receptor chain usage. For example, the dual functions of OSM, another type 1 cytokine family member associated with both pro- and anti-inflammatory functions, have largely been attributed to the usage of two distinct signaling receptors in humans: LIFRβ/gp130 or OSMRβ/gp130 heterodimers (Chen and Benveniste, 2004). Using a panel of BaF3 cell lines expressing IL-31Rα in combination

with gp130, IL-12Rβ1, IL-12Rβ2, IL-27Rα, IL-23R, or OSMR, Dillon et al determined that IL-31 likely signals only through IL-31Rα-OSMR heterodimers (Dillon *et al.*, 2004b). However, it is still unclear whether IL-31Rα could heterodimerize with other family members *in vivo* to form a signaling receptor for additional ligands. In addition, similar to gp130, IL-31Rα has multiple splice variants and a putative soluble receptor, although the functional significance of these is unknown. Thus, determining tissue distribution of potential dimerization partners and receptor variants may be critical to understanding the functional biology of IL-31R signaling.

Differential effects of IL-31R signaling in distinct tissues may also be the result of distinct patterns of both receptor expression and dominant signaling pathways downstream of receptor ligation. IL-31R ligation results in the activation of multiple JAK-STAT and MAPK signaling pathways and the most prominent STAT activated following IL-31R ligation, STAT3, has been shown to mediate diverse effects dependent upon target cell type (Akira, 2000). Recent studies of other members of the type 1 cytokine family have also highlighted the ability of these cytokines to mediate pleiotropic effects depending on the cytokine milieu in which they are expressed or, in infection models, the nature of the pathogen used. The IL-27-IL-27R signaling pathway for example is another gp130 family member that can differentially regulate inflammatory responses in multiple disease settings. IL-27 can function to both promote Th1 cell responses and limit Th2 responses during *Leishmania major* infection (Artis *et al.*, 2004a; Yoshida *et al.*, 2001) and can inhibit Th2 cytokine-dependent inflammation associated with mouse models of asthma and *Trichuris* infection (Miyazaki *et al.*, 2005; Artis *et al.*, 2004c). IL-27 has also recently been shown to inhibit IL-17 responses and promote IL-10 production depending

on the cytokine milieu in which it is expressed (Stumhofer *et al.*, 2006). However, the IL-31-IL-31R pathway is unique in at least two aspects. First, IL-31 is expressed by differentiated $CD4^+$ Th2 cells while IL-27 is expressed by antigen presenting cells. Second, unlike IL-27-IL-27R interactions, IL-31R-mediated suppression appears to be specific for Th2 cytokine responses since deficiency in IL-31Rα did not influence Th1 cell differentiation *in vitro* or the magnitude of the Th1 cytokine response to intracellular pathogens *in vivo*. This specificity in function highlights the potential of manipulating the IL-31-IL-31R signaling pathway in the treatment of inflammatory diseases characterized by over-expression of Th2 cytokines at mucosal sites.

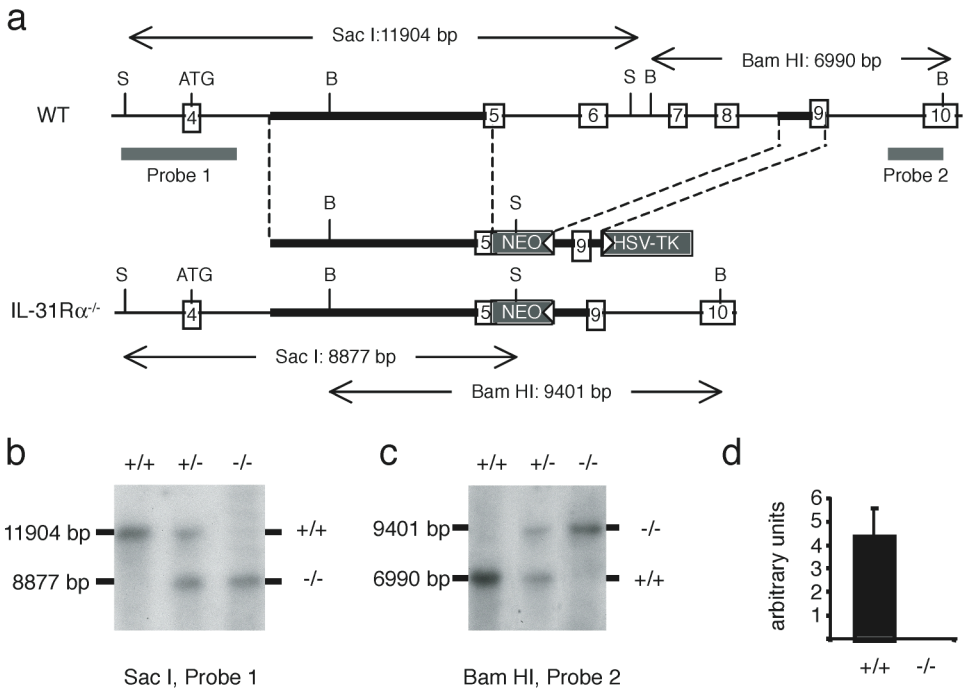

Figure 21. Generation of IL-31Rα$^{-/-}$ mice. (a) The relevant section of the native IL-31Rα locus (top), the targeting construct (middle), and the correctly targeted locus (bottom) are depicted. Open boxes with numbers indicate exons and grey boxes indicate the selection genes neomycin phosphotransferase (neo) and thymidine kinase (tk), with white arrowheads depicting their transcriptional orientation. Thick black lines indicate the position of the 5'- and 3'-homology arms. The size of restriction fragments resulting from digestion with SacI (S) and BamHI (B) are indicated and the location of the two probes used to detect these fragments by Southern blot are shown by thick grey lines. (b-c) Southern blot analysis of SacI digests probed with probe 1, and BamHI digests probed with probe II, respectively. Genotype is indicated as follows: +/+ (WT), +/-

(heterozygous), -/- (IL-31R$\alpha^{-/-}$) (d) Expression of IL-31Rα relative to rpl19 was determined by quantitative Taqman PCR.

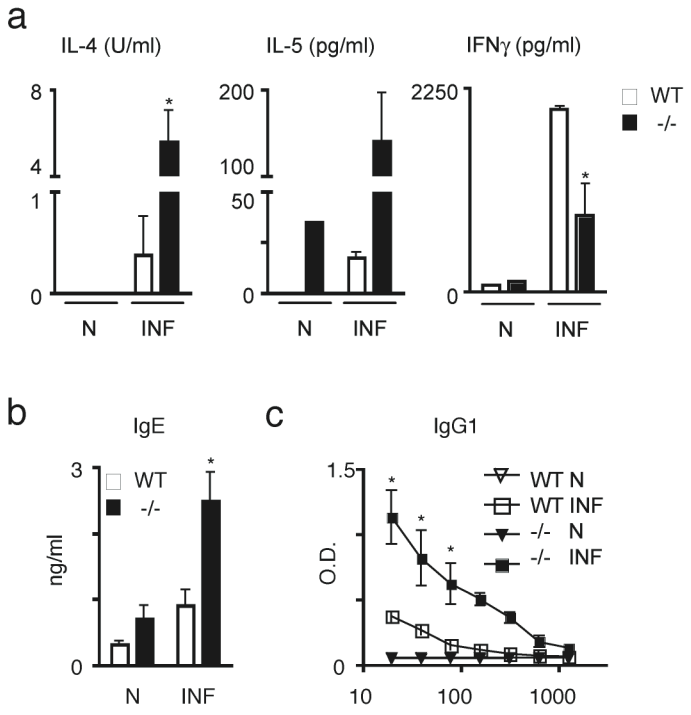

Figure 22. IL-31Rα$^{-/-}$ mice exhibit enhanced Th2 cytokine and Ig responses following infection with *Trichuris*. (a) WT and IL-31Rα$^{-/-}$ mice were infected with 200 *Trichuris* eggs orally and sacrificed on day 18 post-infection. mLNs from naive or infected mice were harvested and cultured *in vitro* in the presence of 1ug/ml anti-CD3/anti-CD28 for 48 hours. Supernatants were assayed by ELISA for IL-4, IL-5, and IFN-γ. (b-c) Serum from *Trichuris* infected WT and IL-31Rα$^{-/-}$ mice at day 18 post-infection was assayed for total IgE (b) and antigen-specific IgG1 antibodies (c) by ELISA. Open bars = WT mice, filled bars = IL-31Rα$^{-/-}$ mice. Results are representative of three independent experiments with 3-4 mice per group. * p<0.05.

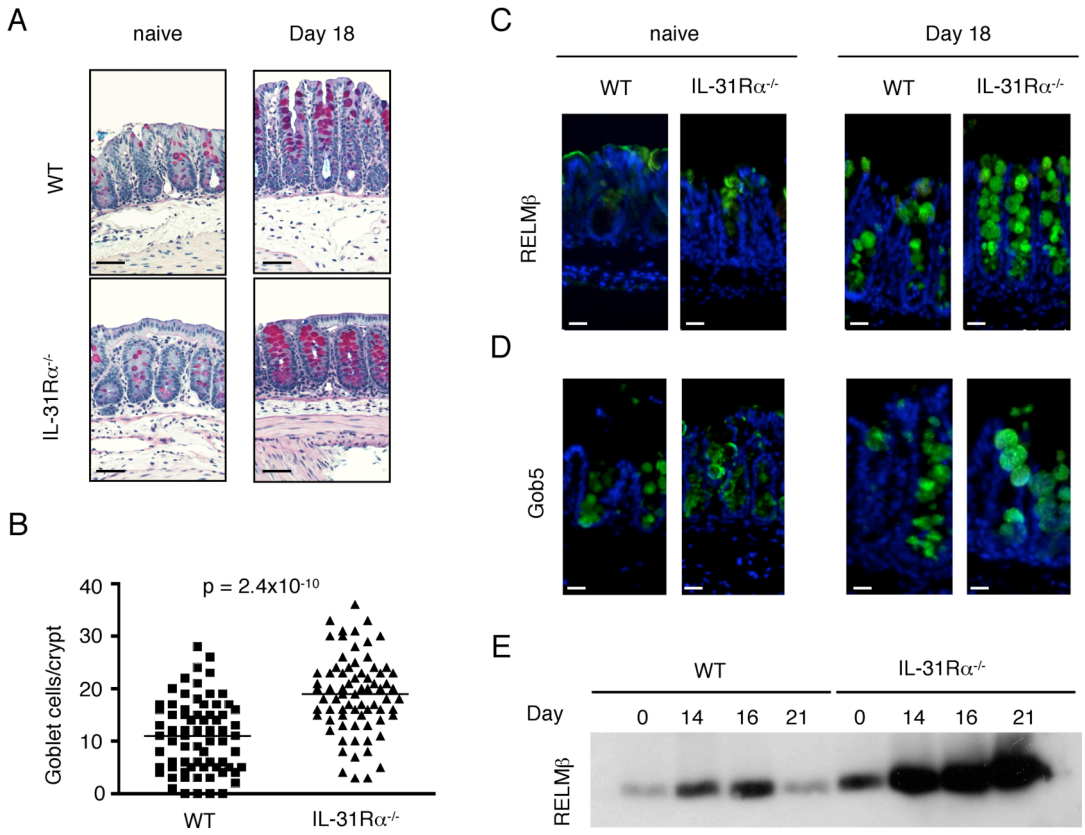

Figure 23. Enhanced expression of goblet cell markers in *Trichuris*-infected IL-31Rα$^{-/-}$ mice. (a) Cecal sections from naive and day 18 infected WT and IL-31Rα$^{-/-}$ mice were stained with Periodic Schiff's/Alcian blue for mucins. (b) Quantification of the number of goblet cells per crypt in day 18 infected WT and IL-31Rα$^{-/-}$ mice. (c-d) Cecal sections from naive and day 18 infected WT and IL-31Rα$^{-/-}$ mice were stained with antibodies against goblet cell proteins RELMβ (c) or Gob5 (d) (shown in green; DAPI nuclear stain in blue). (e) Protein was extracted from pooled fecal pellets obtained at days 0, 14, 16, and 21 post-infection and 30μg analyzed by Western blot to assess lumenal secretion of RELMβ. Results are representative of three independent experiments with 3-4 mice per group.

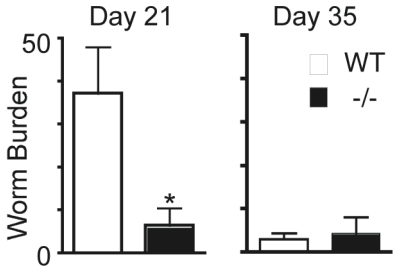

Figure 24. Accelerated worm expulsion in IL-31R$\alpha^{-/-}$ mice. Cecal worm burdens of infected WT and IL-31R$\alpha^{-/-}$ mice were determined microscopically at days 21 and 35 post-infection. Open bars = WT mice, filled bars = IL-31R$\alpha^{-/-}$ mice. Results are representative of four independent experiments with 3-4 mice per group.

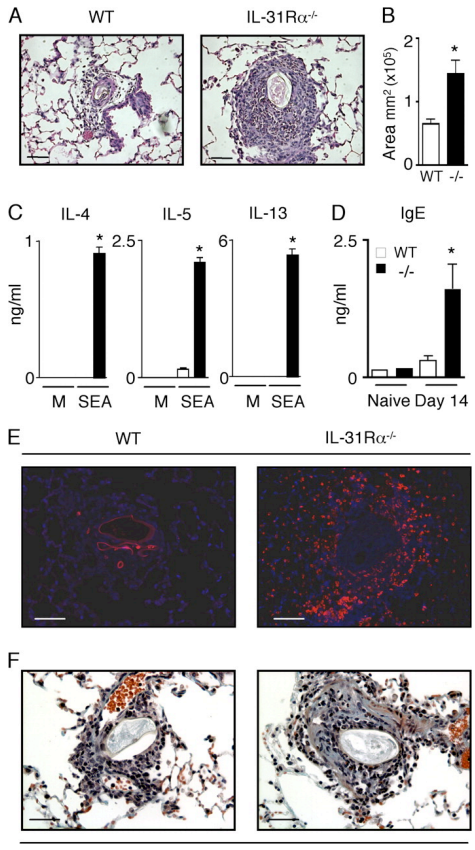

Figure 25. Enhanced *S. mansoni* egg-induced type 2 inflammation in the lungs of IL-31Rα$^{-/-}$ mice. (a-b) Paraffin sections of lungs from egg-injected mice at day 14 post-injection were H&E stained and the area of inflammation surrounding WT (open bar) and IL-31Rα$^{-/-}$ (filled bar) *S. mansoni* egg-induced granulomas was measured using OpenLab software. Mean +/- SEM n=40 granulomas per group. (c) Draining mediastinal lymph node cells from egg-injected WT and IL-31Rα$^{-/-}$ mice were stimulated with either media or 20 μg/ml SEA for 48 hours. Supernatants were analyzed by ELISA for IL-4, IL-5, and IL-13. Mean +/- SEM of replicate cultures. (d) Serum levels of IgE from WT and IL-31Rα$^{-/-}$ mice were measured by ELISA. Mean +/- SEM, n=three mice per group. (e) Immunofluorescence staining for RELM-α (red) and counterstain for DAPI (blue). (f)

Collagen deposition in the lungs of WT and IL-31R$\alpha^{-/-}$ mice was detected by Masson's trichrome staining; blue staining demarks collagen. Bar is 60μm in panels a,e,f. Results are representative of three independent experiments of three mice per group; * indicates statistically significant as determined by two-tailed Students *t* test ($p<0.05$).

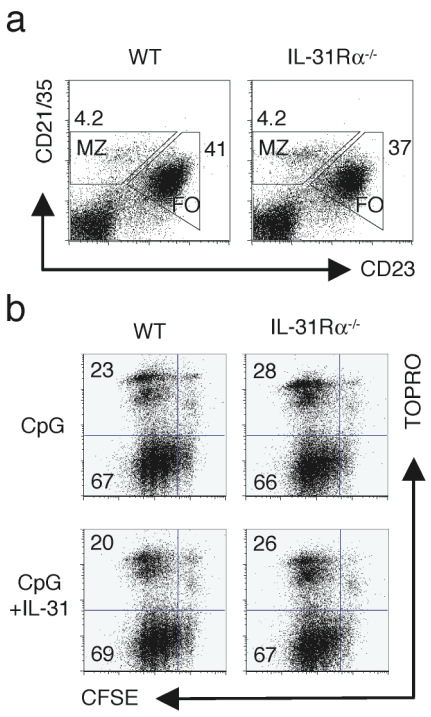

Figure 26. WT and IL-31Rα$^{-/-}$ B cells exhibit equivalent proliferation and survival *in vitro*. (a) Splenocytes were isolated from WT and IL-31Rα$^{-/-}$ mice and stained with antibodies against CD21 and CD23 to determine percentages of follicular (FO) and marginal zone (MZ) B cells. (b) Purified CD23^{+} B cells were CFSE-labeled, plated onto a 96-well plate, and stimulated with CpG (0.1μM) in the presence or absence of IL-31 (50ng/ml) as indicated. After four days in culture, B cells were harvested, stained with TOPRO, and analyzed by flow cytometry. Results are representative of two independent experiments.

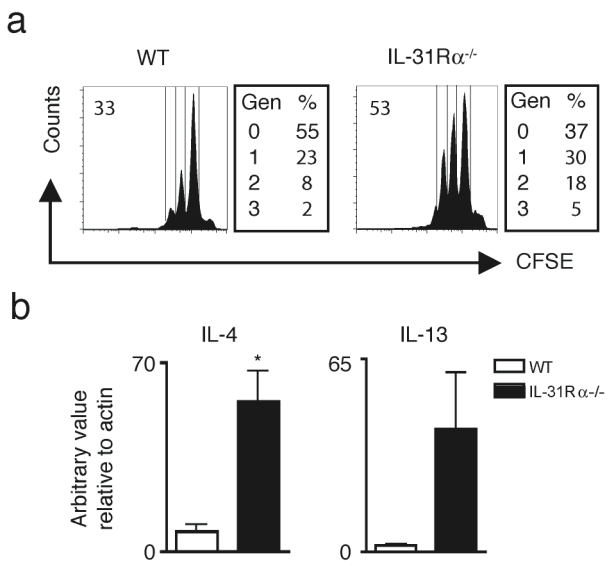

Figure 27. Naive IL-31Rα$^{-/-}$ CD4^{+} T cells exhibit enhanced proliferation and expression of Th2 effector cytokines. (a) Purified naive CD4^{+} T cells from WT or IL-31Rα$^{-/-}$ mice were CFSE labeled and stimulated with plate-bound anti-CD3/anti-CD28 for four days under neutral conditions. Boxes to the right indicate the percentage of events in each proliferating generation (Gen). Numbers in italics in upper left indicate percentage of CFSE-dim cells. (b) cDNA derived from the cells described above was assayed by real-time PCR for IL-4 or IL-13 mRNA levels relative to actin. Results expressed as the mean +/- SEM for three independent experiments. * $p<0.05$.

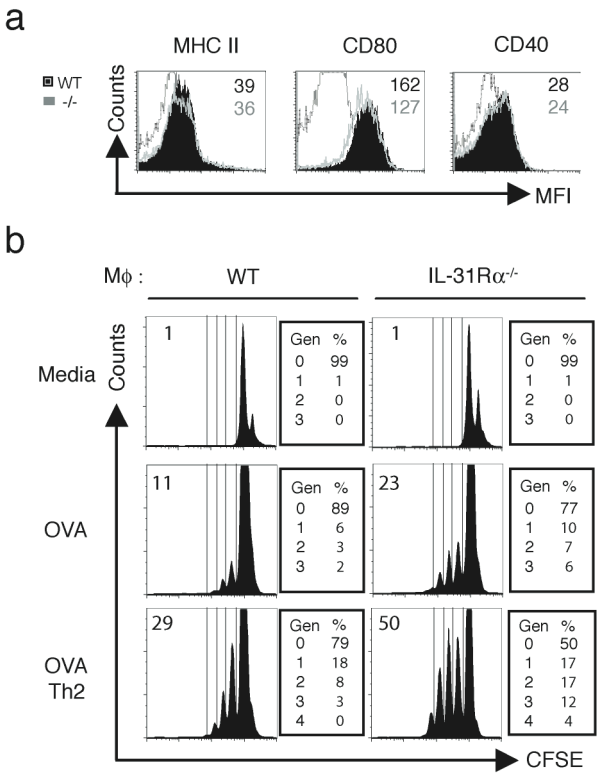

Figure 28. IL-31Rα$^{-/-}$ macrophages exhibit enhanced accessory cell function. (a) Bone marrow-derived macrophages from either WT (filled black) or IL-31Rα$^{-/-}$ (grey line) mice were characterized for surface molecule expression of MHC II, CD80, or CD40. Numbers refer to MFI (black, WT; grey, IL-31Rα$^{-/-}$). Black line is isotype control. (b) WT or IL-31Rα$^{-/-}$ macrophages were pulsed overnight with OVA protein in the presence or absence of IL-4 when indicated. CFSE-labeled OTII T cells were added twenty-four hours after OVA pulse and co-cultured with macrophages for four days under neutral or Th2-polarizing conditions. Histograms are gated on the live CD4^{+} population; boxes to the right indicate the percentage of events in each proliferating generation (Gen).

Numbers in italics in upper left indicate percentage of CFSE-dim cells. Data are representative of six independent experiments.

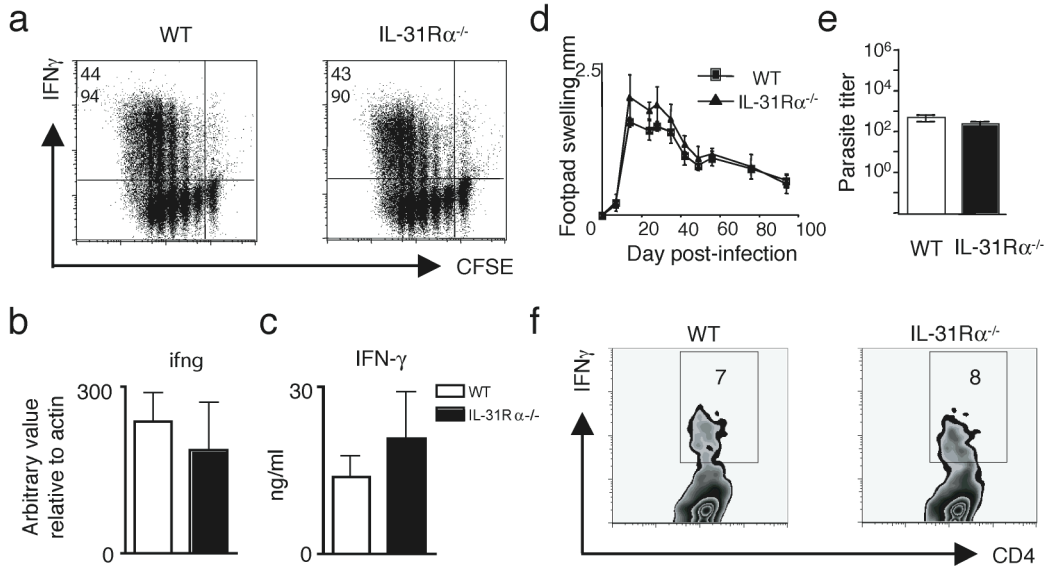

Figure 29. Normal Th1 cell differentiation in IL-31Rα$^{-/-}$ mice. (a) Purified CD4^{+} T cells from WT and IL-31Rα$^{-/-}$ mice were CFSE-labeled and stimulated with plate-bound anti-CD3/anti-CD28 under Th1 polarizing conditions. Cells were assayed for proliferation and cytokine production by flow cytometry. The top number indicates the percentage of proliferating CD4^{+} T cells producing IFN-γ and the bottom number in italics is the percentage of CFSE-dim cells. (b) cDNA from purified naive CD4^{+} T cells cultured under Th1-polarizing conditions was subjected to real-time PCR analysis for IFN-γ mRNA expression relative to actin. (c) Supernatants from the cultures in (a) were assayed by ELISA for IFN-γ levels. (d) WT and IL-31Rα$^{-/-}$ mice were infected with *L. major*

promastigotes in the hind footpad. Lesion size was determined by measuring swelling of the infected footpad and subtracting that of the uninfected contralateral footpad. (e) Parasite load in the footpad was quantified by limiting dilution analysis at 12 weeks post-infection. Values represent the mean +/- SEM for three mice per group. (f) Cells from the draining lymph nodes of *L. major* infected WT or IL-31R$\alpha^{-/-}$ mice were re-stimulated with soluble *Leishmania* antigen and analyzed by flow cytometry. Plots are gated on $CD4^+$ cells and the number in the box indicates the percentage of $CD4^+$ T cells producing IFN-γ.

	Spleen			Lymph node		
	WT	KO	P	WT	KO	P
CD4+ (% of live)	18.5 +/- 2.0	18.7 +/- 1.5	0.878	38.8 +/- 3.0	37.3 +/- 6.2	0.688
CD25+ (% of CD4+)	11.1 +/- 0.8	12.3 +/- 1.9	0.288	13.0 +/- 0.9	11.3 +/- 2.3	0.224
CD62L+ (% of CD4+)	73.1 +/- 5.8	69.2 +/- 1.5	0.248	89.4 +/- 1.4	88.5 +/- 4.7	0.718
CD8+ (%of live)	10.6 +/- 2.7	9.7 +/- 0.3	0.520	28.9 +/- 2.7	28.9 +/- 2.7	0.353
CD62L+ (% of CD8+)	84.4 +/- 3.6	80.9 +/- 1.5	0.165	94.3 +/- 1.3	94.5 +/- 2.3	0.853
CD11b+CD11c+	3.2 +/- 1.2	3.8 +/- 0.6	0.414	0.3 +/- 0.1	0.7 +/- 0.3	0.062
CD11b+CD11c-	8.0 +/- 2.3	8.8 +/- 1.5	0.552	0.9 +/- 0.2	2.3 +/- 1.3	0.076
CD11c+CD11b-	4.0 +/- 1.6	3.4 +/- 0.8	0.578	0.5 +/- 0.1	0.6 +/- 0.1	0.211
F4-80+	11.3 +/- 3.0	12.9 +/- 0.5	0.330	0.8 +/- 0.1	2.2 +/- 1.1	0.037
B220+	55.6 +/- 4.0	55.6 +/- 6.2	0.995	28.2 +/- 8.5	24.4 +/- 3.5	0.442
NK	2.4 +/- 0.2	2.4 +/- 0.3	0.878	0.5 +/- 0.2	0.6 +/- 0.1	0.727
NKT	0.8 +/- 0.1	0.7 +/- 0.2	0.406	0.4 +/- 0.1	0.6 +/- 0.2	0.085

	Thymus		
	WT	KO	P
CD4-CD8+	11.8 +/- 2.7	8.8 +/- 2.2	0.130
CD4+CD8+	74.0 +/- 8.7	79.0 +/- 5.9	0.375
CD4+CD8-	8.2 +/- 2.1	7.6 +/- 2.3	0.722
CD4-CD8-	6.1 +/- 4.1	5.6 +/- 5.5	0.900

Table 1. Normal immune cell composition of thymus, spleen and peripheral lymph nodes in IL-31R$^{-/-}$ mice.

Chapter 5
Summary, Discussion and Future directions

5.1 Cooperative mechanisms for Th2 cell development

It has been 23 years since Coffman and Mosmann first defined two types of helper T cell clones with distinct phenotypic and functional attributes and 20 years since Janeway first proposed the theory of pattern recognition. The intersection of these two ideas, that innate immune cells can recognize and respond to unique classes of pathogens and direct the differentiation of divergent protective effector T helper cell fates, has become the paradigm for how the mammalian host generates protective adaptive immunity to pathogenic microbes. DCs are central players in this paradigm capable of recognizing pathogens through expression of a wide range of pattern recognition receptors (PRRs). Ligation of PRRs by viral, bacterial, fungal or protozoan products induces increases in DC MHC class II and co-stimulatory molecule expression to initiate the activation of naïve T cells and, depending on the context of stimulation and subset of DC, the production of multiple soluble mediators including TGF-β, IL-6, IL-12 and IL-23 that promote Th1 and Th17 cell differentiation (see **Chapter 1, Section 1.2.3**). In contrast, exposure of DCs to helminth antigens does not induce their phenotypic maturation and few DC-derived factors that promote the development of Th2 cell differentiation have been identified (MacDonald and Maizels, 2008). Additionally, the inability to identify conserved PAMPs or PRRs devoted to the recognition of helminth parasites has provoked speculation that the current paradigm of pattern recognition may not apply to this class of pathogens and other biochemical mechanisms of innate immune cell activation may be involved (Medzhitov, 2009). Thus, the role of DCs in the development

of protective Th2 cell responses has remained unclear. Within this context, one of the goals of this thesis was to interrogate the innate cellular requisites for the initiation of Th2 cell-dependent immunity and inflammation.

In **Chapter 2**, I directly tested the hypothesis that antigen presentation by $CD11c^+$ DCs would be sufficient to initiate Th2 cell differentiation and protective immunity to the helminth *Trichuris* using mice in which MHC class II expression was restricted to $CD11c^+$ cells (MHC II^{CD11c} mice). From these studies, we demonstrated that $CD11c^+$ DC-restricted MHC class II expression was not sufficient for the generation of protective Th2 cytokine-dependent immunity and that in the absence of other non-$CD11c^+$ APC-T cell interactions, a non-protective Th1 cytokine response emerged. Current models of Th2 cell differentiation *in vivo* favor priming of naïve T cells by DCs coupled with provision of soluble Th2-polarizing factors, such as IL-4, IL-25, IL-33 and TSLP, by other innate cells such as basophils, eosinophils, mast cells, IEC and NKT cells to enforce Th2 cell fate commitment. However, our data suggest that cognate MHC class II-dependent interactions between T cells and a non-$CD11c^+$ population are required for Th2 cytokine-dependent immunity to *Trichuris* and suggest that other APCs may play a dominant role in Th2 cell differentiation *in vivo*. Consistent with this hypothesis, transient depletion of $CD11c^+$ DCs throughout the course of *Trichuris* infection using CD11c-DTR bone marrow chimeras did not alter Th2 cytokine responses or worm burdens and although there was a reduction in the population expansion of $CD4^+$ T cells, on a per cell basis they secreted elevated levels of Th2 cytokines. Consistent with our observations, Medzhitov and colleagues showed that restricting antigen presentation to $CD11c^+$ DCs was insufficient for the development of a Th2 cytokine response after exposure to the

protease allergen papain and depletion of $CD11c^+$ DCs did not alter the development of papain-induced Th2 cell responses *in vitro* or *in vivo* (Sokol *et al.*, 2009). Further, this study demonstrated that papain likely gains direct access to the lymph and is transported to APCs within the lymph node without requiring acquisition by migratory DCs since removal of the tissue surrounding the injection site did not abolish Th2 cell differentiation (Sokol *et al.*, 2009). Collectively these data indicate that $CD11c^+$ DCs are neither necessary nor sufficient for the development of Th2 cytokine responses following exposure to papain or infection with the helminth *Trichuris*.

However, this is not to say that DCs do not contribute to Th2 cell-dependent immunity and inflammation or that they cannot promote Th2 cell differentiation in other settings. For example, as discussed in **Chapter 1**, **Section 1.3**, DCs conditioned with *S. mansoni* egg antigen preparations have clearly been shown to promote Th2 cell differentiation both *in vitro* and *in vivo* and components of SEA can modulate multiple facets of DC function. SEA treatment renders DCs refractory to subsequent TLR stimulation while actively promoting antigen processing and presentation in the absence of other classical signs of maturation (Marshall and Pearce, 2008). A recent study has also identified a component of SEA called Omega1, a T2 ribonuclease that can limit the ability of DCs to form stable conjugates with T cells and mimick low antigen dose TCR stimulation that is associated with Th2 cell differentiation (Steinfelder *et al.*, 2009). However, despite the evidence that SEA-stimulated DCs can promote Th2 cell responses, whether they are necessary or sufficient to do so *in vivo* during *S. mansoni* infection is still unknown.

The studies carried out with SEA and other helminth antigen preparations provoke the hypothesis that the components within excretory/secretory (ES) products derived from distinct species of helminth parasites may dictate a requirement for non-DC APCs to promote protective Th2 cell-dependent immunity. This point is illustrated by a recent report demonstrating that the requirement for TSLP in the development of Th2 cell-dependent immunity to *Trichuris* may, in part, be due to the fact that unlike ES from *H. polygyrus* or *N. braziliensis*, *Trichuris* ES cannot limit IL-12 production from DCs (Massacand *et al.*, 2009). Consistent with the idea that additional APC-T cell interactions are required to limit Th1 cell differentiation during *Trichuris* infection, blockade of IFN-γ in MHC II^{CD11c} mice recovers Th2 cell responses and immunity (**Chapter 2, Fig. 11**). Taking these findings together I would predict that the requirement for additional non-DC APCs to either inhibit Th1 cell differentiation and/or actively promote Th2 cell differentiation through MHC class II-dependent interactions with CD4$^+$ T cells is likely in part dependent on the context of antigenic stimulation and nature of the helminth parasite.

Allergens can also elicit polarized Th2 cytokine responses and DCs have been shown to be involved in allergen sensitization. In a murine model of asthma for example, intratracheal instillation of DCs pulsed with OVA can sensitize mice to airway hyper-responsiveness and depletion of DCs during OVA airway challenge results in decreased Th2 cytokine production and the abrogation of lung pathology (van Rijt *et al.*, 2005). However in response to the protease allergen papain, it is clear that DCs do not play a critical role in the development of Th2 cytokine responses as they are neither sufficient nor necessary to promote Th2 cell differentiation *in vitro* or *in vivo* (Sokol *et al.*, 2009).

There are many potential reasons for the differential requirements for DCs in these models. One important difference in these models is the site of antigen exposure. As discussed previously, DCs isolated from mucosal sites have been shown to preferentially induce Th2 cell responses and the introduction of allergens via intratracheal as opposed to intradermal routes is likely to have a substantial impact on the resulting immune response. Additionally, as with helminth ES products, there is some diversity in the biochemical activities of allergens and they can be divided into those that elicit Th2 cytokine responses in a protease-dependent or -independent manner. Therefore, the role of distinct APC populations in the promotion of type 2 inflammatory responses to allergens could be dictated by lineage-specific expression of protease-activated receptors. Taken together, I believe these data demonstrate that there are likely multiple pathways leading to Th2 cell differentiation, some of which are DC-independent, depending on the nature of the antigen, the site of antigen exposure and whether concomitant Th1 cell differentiation needs to be repressed. Further studies will be required to directly assess the role of DCs in promoting Th2 cell responses to other allergens and helminth infection models and to identify novel pathways that mediate the recognition of these diverse Th2 cell-promoting stimuli.

5.2 Basophils as APCs for Th2 cell differentiation

The data discussed above indicated that additional non-DC APCs are required for the promotion of Th2 cell differentiation *in vivo* following exposure to some gastrointestinal helminths and protease allergens. We demonstrated in **Chapter 2** that B cells and macrophages did not play critical, non-redundant roles in promoting type 2 immunity to *Trichuris*, provoking a search for non-professional APCs that may be involved in Th2 cell

differentiation. In **Chapter 3**, I identified a previously unappreciated function for basophils as APCs following exposure to helminths and demonstrated that depletion of basophils impaired immunity to *Trichuris*. In an acute model of helminth-induced Th2 cell differentiation, exposure to *S. mansoni* eggs resulted in the rapid and transient recruitment of MHC class II^+ basophils to the draining lymph node and adoptive transfer of basophils could augment egg-induced $CD4^+$ Th2 cell responses. In addition, purified basophils expressed MHC class II and IL-4 message and could promote the proliferation and Th2 cell differentiation of antigen-specific $CD4^+$ T cells *in vitro*. Parallel studies from Medzhitov and colleagues and Nakanishi and colleagues also demonstrated that basophils could endocytose soluble antigen and IgE-allergen complexes, express MHC class II and co-stimulatory molecules and promote the differentiation of Th2 cells *in vitro* and *in vivo* (Sokol *et al.*, 2009; Yoshimoto *et al.*, 2009). Antigen-pulsed basophils were sufficient to promote Th2 cell differentiation following adoptive transfer to CIITA-deficient hosts, suggesting that antigen-transfer from injected basophils to other APCs *in vivo* could not account for the development of Th2 cell responses (Sokol *et al.*, 2009). Together, these data are the first demonstration that basophils are not only capable of augmenting Th2 cell responses through production of soluble factors such as IL-4, IL-13 and TSLP but can directly prime antigen-specific Th2 cell responses. These data provide the foundation for a number of areas for future study including determining the signals responsible for mediating the recruitment of basophils to draining lymph nodes, how basophils recognize helminths and allergens and what factors regulate basophil APC function.

The emerging central role for basophils in the development and maintenance of Th2 cytokine-dependent immunity and inflammation has thus also resulted in a renewed interest in the factors that govern basophil development. The contribution of IL-3 to the development as well as helminth-induced expansion of basophils is well established. However, in **Chapter 3** we identified a novel role for the IL-7-like cytokine TSLP in promoting peripheral basophilia. In addition, while we did not observe increases in peripheral basophil frequency following treatment with recombinant IL-33, another epithelial cell-derived cytokine, a recently published study has demonstrated that IL-33 can promote basophil expansion in the bone marrow through increased production of IL-3 and GM-CSF (Schneider *et al.*, 2009). Collectively, these data identify two cytokines predominantly expressed within the epithelium with the potential to influence basophil responses. It is thus tempting to speculate that early activation of IECs following exposure to gastrointestinal helminths such as *Trichuris* or allergens may contribute to the activation, proliferation or perhaps recruitment of basophils during helminth infection. Similarly, recent data has shown that proteases, including papain, can stimulate TSLP production from epithelial cells in the skin and lung (Briot *et al.*, 2009; Kouzaki *et al.*, 2009) implicating the epithelium as a primary target for allergens with protease activity. The ability of diverse cytokines to promote basophilia also provokes the question as to whether cytokine-elicited basophils represent phenotypically distinct populations. Preliminary data from our lab suggests that this may be the case. For example, bone marrow-derived TSLP-elicited basophils exhibit higher levels of MHC class II expression than IL-3-elicited basophils (personal communication, Mark Siracusa). Whether these differences in surface phenotype translate to changes in functional capacity to promote

Th2 cell differentiation has yet to be determined and these are ongoing studies in the lab.

The ability of IgE-antigen complexes to activate basophils via FcεRI ligation has long been appreciated. However, a recent study has now demonstrated that human basophils selectively bind IgD, a class of antibody produced early in B cell development and conserved from jawed fish to mammals (Chen *et al.*, 2009). The biological function of IgD has remained unclear but this study demonstrates that IgD is highly expressed in the human upper respiratory tract, can bind the respiratory bacteria *Haemophilus influenzae* and *Moraxella catarrhalis* and can activate basophils to produce anti-microbial peptides that inhibit bacterial replication. IgD activation of basophils also resulted in a distinct effector phenotype from that of IgE activation; in comparison with IgE crosslinking, IgD crosslinking resulted in increased expression of IL-4, B cell activating factor (BAFF), broad spectrum antimicrobial peptides and had no effect on histamine release (Chen *et al.*, 2009). IgD was also efficiently internalized by basophils, suggesting that analogous to basophil uptake of IgE-antigen complexes, IgD-antigen complexes could be internalized and presented via MHC class II for presentation to T cells, however this hypothesis has yet to be tested.

Another critical question from our studies is how helminth-derived products and protease allergens activate basophils and promote APC function. Protease-activated receptors (PARs) are prime candidate receptors that may be activated in response helminth-derived proteases or protease allergens such as papain. Of particular interest is the

PAR2 receptor that is expressed on a wide range of hematopoietic and non-hematopoietic cells and has recently been shown to induce the expression of TSLP from epithelial cells (Briot *et al.*, 2009; Kouzaki *et al.*, 2009). However, human basophils lack expression of any PAR family members (Falcone *et al.*, 2005) and there is no evidence for PAR expression in murine basophils, suggesting that basophils may express other PAR-like receptors. In fact, the lack of PAR expression on basophils is conspicuous in that nearly all other myeloid cells including neutrophils, eosinophils, mast cells, macrophages and DCs have been shown to express PARs (Shpacovitch *et al.*, 2008). Of note, basophils also appear to selectively express an unknown receptor responsible for binding IgD and treatment of basophils with the proteases papain or trypsin abrogated IgD binding, indicating that basophil recognition of IgD and papain may involve a common receptor (Chen *et al.*, 2009).

The list of stimuli that promote basophil activation includes cytokine stimulation, exposure to helminths and protease allergens, FcεRI ligation and now IgD signaling (**Fig. 30**). However, our knowledge of the negative regulatory pathways that constrain basophil function is less clear and may be critical in the development of new treatments for Th2 cytokine-dependent inflammation associated with asthma and allergy. Recent progress in this area has been aided in part through reassessment of knock-out mice that develop spontaneous type 2 inflammation. For example, interferon regulatory factor 2 (IRF-2) deficient mice exhibit Th2-skewing and preferentially develop Th2 cytokine responses following infection with the parasite *Leishmania major* (Lohoff *et al.*, 2000). While early reports suggested that impaired production of IL-12/23p40 by macrophages contributed to this phenotype, it was recently demonstrated that basophil populations in

the spleen and blood were significantly increased in IRF-2$^{-/-}$ mice compared to WT mice and implicated IRF-2 in the negative regulation of IL-3 signals that normally promote basophil development (Hida *et al.*, 2005). Similarly, mice deficient in the signaling molecule Lyn kinase develop spontaneous atopic allergic responses early in life and a recent report has linked Lyn deficiency with exacerbated basophil responses (Charles *et al.*, 2009). The results of that study demonstrate that the Lyn kinase pathway, activated downstream of FcεRI signaling, negatively regulates basophil proliferation as well as inhibits basophil expression of IL-4 and the transcription factor GATA3 (Charles *et al.*, 2009). These effects were proposed to be mediated by potential interplay between Lyn kinase and Fyn kinase, an activator of the PI3 kinase pathway associated with increased GATA3 expression and enhanced IL-4 production in T cells. Consistent with these data, another recently published study has identified a role for SHIP, a negative regulator of the PI3 kinase pathway, in the inhibition of IL-4 production from basophils (Kuroda *et al.*, 2009). Loss of SHIP led to an increase in the development of IL-3-induced alternatively activated or "M2" macrophages due to enhanced IL-4 production from basophils, suggesting that basophils may influence innate immune cell differentiation pathways as well as adaptive CD4^{+} T cell differentiation. Collectively, these studies indicate that manipulation of the PI3 kinase pathway in basophils could influence multiple aspects of the development of Th2 cytokine-dependent immunity and inflammation.

As discussed above, the intracellular pathways that inhibit basophil function are beginning to be elucidated, however few surface inhibitory receptors have been identified on basophils. One potential inhibitory receptor is the FcγRIIb, a prototypic ITIM-containing receptor that recruits SHIP and is required to inhibit IgG-mediated

anaphylaxis (Takai *et al.*, 1996), an experimental model recently shown to be critically dependent on basophils (Tsujimura *et al.*, 2008). Additionally, a recent report has identified the surface receptor CD200R as a potential marker for activated basophils (Torrero *et al.*, 2009). CD200R is the prototypic member of a family of receptors that may be analagous to the paired activating and inhibitory receptors on NK cells. While CD200R ligation is associated with inhibitory signaling, other members of the family such as CD200R3 can deliver activating signals through adaptor molecules like DAP12. However, DAP12$^{-/-}$ mice develop normal Th2 cell responses following infection with *Nippostrongylus braziliensis*, suggesting that either the activating CD200R3 receptor is not required for basophil function or that other adaptors compensate for loss of DAP12 (Voehringer *et al.*, 2004b). Ligation of the inhibitory receptor CD200R has previously been shown to suppress the release of histamine from basophils, however its ability to limit cytokine production and other aspects of basophil function have not been assessed (Shiratori *et al.*, 2005). Phosphorylation of CD200R results in the recruitment of Dok1 and Dok2 to its cytoplasmic domain and subsequent interaction with RasGAP and SHIP. Given the association between SHIP and the negative regulation of basophil IL-4 production discussed above, treatment with agonists of CD200R may be a mechanism to selectively inhibit basophil function in type 2 inflammatory settings. Of note, mice deficient in CD200, the ligand for CD200R, have been shown to develop enhanced Th2 cytokine responses to antigen in the absence of adjuvant (Taylor *et al.*, 2005). In that study, splenocytes from CD200$^{-/-}$ mice that received an intranasal administration of antigen exhibited significantly increased levels of antigen-specific IL-4 and IL-5 compared to WT controls, suggesting that CD200-CD200R interactions are critical in the regulation of Th2 cytokine responses under homeostatic conditions. CD200 is

expressed constitutively in the epithelium of mucosal sites such as the lung and the cytokines IL-10 and TGF-β, both expressed at elevated levels at mucosal sites, have been shown to upregulate CD200R expression on myeloid cells. Thus, CD200-CD200R interactions are proposed to be a way in which to regulate the threshold of activation of tissue resident cells and maintain local tolerance at mucosal sites (Snelgrove *et al.*, 2008). Given that a previous study has demonstrated that CD200R expression levels within basophils may be up to 100 times that of other myeloid or lymphoid cell populations (Shiratori *et al.*, 2005), CD200R ligation represents an appealing target for treatment of Th2 cytokine-dependent inflammatory diseases. We are currently evaluating effects of various stimuli on basophil expression of CD200R.

Our data demonstrating a role for basophils as APCs that can promote Th2 cell development is consistent with the large body of literature identifying basophils as key players in the promotion of type 2 immunity and inflammation. However, there is some data to suggest that basophils may also serve a regulatory role in limiting inflammation. For example, basophil co-culture with $CD4^+$ T cells was shown to limit IFN-γ production in a partially contact-dependent manner indicating that basophil-$CD4^+$ T cell interactions may be involved in the inhibition of Th1 cell differentiation, although the factors that mediate this effect are unknown (Oh *et al.*, 2007). Additionally, a recent study has demonstrated that basophils can present and cross-present soluble antigens to $CD8^+$ T cells and promote their differentiation into Tc2-like, IL-10-producing $CD8^+$ T cells (Kim *et al.*, 2009). Although the IL-10-producing $CD8^+$ T cells in that study did not have regulatory capacity *in vitro*, they may function to limit inflammation *in vivo*. Basophils may also secrete soluble factors that directly limit inflammatory responses. In human

studies, basophils have been shown to selectively express retinaldehyde dehydrogenase-II (RALDH2) and produce retinoic acid (RA) to promote Th2 cell differentiation and expression of the gut-homing integrin $\alpha4\beta7$ (Spiegl *et al.*, 2008). However, RA production by $CD103^+$ DCs within the intestine has been shown to promote Foxp3 expression in $CD4^+$ T cells, suggesting that basophil-derived RA may have a similar effect. Future studies will be required to determine the functional significance of basophil-derived RA and whether basophils have any protective immune regulatory role *in vivo*.

5.3 Regulation of Th2 cell responses

In the early 1970s, before the theory of pattern recognition and the definition of distinct effector T cell subsets, there was emerging evidence that T cells could also mediate immunoregulatory functions and that these functions may be an essential part of limiting pathogen-induced inflammation. While these early reports of "suppressor T cells" were eventually buried in controversy and inconsistencies (Germain, 2008), the concept of immune suppression has re-emerged. The isolation of Treg cells that can suppress effector cell responses, coupled with the autoimmune phenotypes of both mice and humans with mutations in the Treg differentiation factor Foxp3, have solidified the importance of regulatory responses in the maintainence of immune homeostasis. This regulation is essential at sites such as the lung and intestine, where host tissue is continually bombarded with foreign antigens of both an innocuous and potentially pathogenic nature. Therefore, a second aim of this thesis has been to identify factors that constrain cytokine-dependent inflammation at mucosal sites.

Regulation of effector cell responses is not only necessary to prevent inflammation in the steady state but to limit collateral damage to host tissues during immune responses to pathogens and recent studies have demonstrated the concomitant development of regulatory and effector T cell responses in multiple infectious settings (Belkaid and Oldenhove, 2008). In some cases, effector cells may also self-regulate, independently of Tregs, through their own production of anti-inflammatory mediators. For example, IFN-γ^+ Th1 cells that develop during both *Toxoplasma gondii* infection and in a model of non-healing *Leishmania major* infection are a primary source of IL-10 and contribute to the negative feedback regulation of this effector cell subset (Anderson *et al.*, 2007; Jankovic *et al.*, 2007). This may also be the case for the predominant expression of IL-31 by Th2 cells.

In **Chapter 4**, I identified a regulatory role for IL-31-IL-31R interactions in limiting Th2 cell-dependent immunity and inflammation in the intestine and the lung. Following infection with *Trichuris*, mice deficient in IL-31R signaling exhibited elevated Th2 cytokine responses, systemic increases in IgE and IgG1, enhanced goblet cell responses associated with increased mucin production and RELM-β expression and accelerated worm expulsion compared to WT controls. Similarly, deficiency in IL-31R resulted in increased Th2 cytokine-dependent inflammation in the lung following exposure to *S. mansoni* eggs. *In vitro*, in the absence of T cell-intrinisic IL-31R signaling, stimulation of naïve $CD4^+$ T cells resulted in increased Th2 cytokine mRNA expression and macrophages deficient in IL-31R could promote enhanced proliferation of $CD4^+$ T cells. Collectively, these results suggest that IL-31 expression by $CD4^+$ T cells may function to limit the magnitude of Th2 cytokine responses through IL-31R

signaling on both innate and adaptive immune cells. However, future studies are required to determine the relative contributions of these distinct cell populations in the development of enhanced type 2 inflammation in IL-31R$^{-/-}$ mice.

Our results are consistent with a regulatory role for IL-31-IL-31R interactions in limiting Th2 cytokine-dependent immunity in the lung and intestine following exposure to helminths. However, as discussed in **Chapter 4**, IL-31 belongs to a family of cytokines and receptors with a high degree of pleitropy in function and promiscuity in cytokine and receptor subunit usage. As a heterodimeric receptor, loss of the IL-31Rα component of IL-31R could lead to disruption of signaling through a putative alternative receptor complex or enhanced signaling through other OSMR complexes. Therefore, to further establish the function of IL-31 additional studies *in vitro* and *in vivo* using recombinant IL-31 and antibodies to block endogenous IL-31 activity will be required. Notwithstanding that, our findings indicate a critical role for IL-31R in limiting type 2 inflammatory responses whether through direct IL-31-IL-31R interactions, sequestration of OSMR or signaling through another as yet unidentified receptor complex.

In contrast to our results, previous studies in IL-31 transgenic mice suggested a pro-inflammatory role for IL-31 signaling, specifically in the skin (Dillon *et al.*, 2004b). Overexpression of IL-31 resulted in the development of a pruritic skin condition resembling atopic dermatitis. In **Chapter 4**, I discussed a number of possibilities for this discrepancy including tissue-specific regulation of IL-31R expression or function, differences between overexpression studies and deletion of endogenous receptor and the fact that, like other members of this family, IL-31 may exert both pro- and anti-

inflammatory effects in diverse inflammatory settings. While mutational analyses of the intracellular portion of IL-31Rα have identified residues required for phosphorylation of the receptor (Diveu *et al.*, 2004; Dreuw *et al.*, 2004), additional characterization of the intensity and duration of signaling pathways downstream of receptor ligation may shed light on the functional outcome of signaling. For example, as discussed in **Chapter 1** sustained STAT3 signaling contributes to the anti-inflammatory versus pro-inflammatory functions of IL-10 and IL-6 in macrophages (Lang *et al.*, 2003; Yasukawa *et al.*, 2003). Thus, additional studies examining the lineage-specific effects of IL-31R signaling will be important for deciphering how IL-31R influences innate and adaptive immunity.

While expression is enhanced in Th2 cells, all activated $CD4^+$ T cells induce expression of IL-31, suggesting a potential role for this cytokine in influencing other modules of adaptive immune responses. In **Chapter 4**, we tested the requirement for IL-31R in the development of Th1 responses and found no apparent effect on Th1 cell differentiation *in vitro* or *in vivo*. However, the role of IL-31 in the context of pathogens that induce Th17 cell responses remains to be examined. Although there is no evidence that IL-31-IL-31R signaling can regulate IL-17 production *in vitro* or *in vivo*, this has not been directly tested. IL-31 is structurally similar to IL-6 and IL-31Rα is closely related to gp130 as well as IL-12Rβ2 and IL-23Rα. Coupled with the predominant phosphorylation of STAT3 downstream of IL-31R signaling, there is sufficient rationale to investigate the role of IL-31 in the promotion of Th17 cell responses. In addition, given the involvement of IL-17 in chronic inflammatory skin disease (Pene *et al.*, 2008), the potential for IL-31 to promote IL-17 responses would be another way to reconcile our data with the

dermatitis phenotype associated with transgenic overexpression of IL-31 (Dillon *et al.*, 2004b).

Although outside of the scope of Th2 cell-dependent immunity and inflammation, an additional goal of this thesis was to investigate the functional biology of MHC class II expression by IECs. In **Appendix A** below, we describe the generation of mice with a lineage-specific deletion in IEC-intrinsic MHC class II expression and identify a role for IECs in limiting Th17 cell-dependent inflammation during infection with the enteric pathogen, *Citrobacter rodentium*. In addition, *in vitro* co-culture of IECs with activated $CD4^+$ T cells results in the contact-dependent inhibition of T cell proliferation and cytokine production. Based on these results, we hypothesize that an 'effector synapse' may form between IECs and activated $CD4^+$ T cells within the IEL compartment and lamina propria of the intestine. These interactions may facilitate directed cytokine secretion from the $CD4^+$ T cell towards the epithelium as well as contact-dependent signals from the epithelium to the $CD4^+$ T cell to negatively regulate T cell effector function and limit intestinal inflammation.

5.4 Concluding remarks

The results presented in this thesis provide new insight into the mechanisms of the intiation and regulation of type 2 inflammation at mucosal sites (summarized in **Fig. 31**). Based on the data presented in **Chapters 2 and 3**, a new pathway for Th2 cell differentiation has emerged whereby some pathogens and allergens may be able to promote Th2 cell differentiation via DC-independent, MHC class II-dependent basophil-T cell interactions. Further, our data showing that TSLP can elicit peripheral basophilia

provides another putative link between IEC activation and the initiation of Th2 cell-dependent immunity and inflammation. In **Chapter 4**, we identified a novel negative feedback pathway for the cytokine IL-31 in limiting type 2 inflammation in the intestine and lung. Collectively, these studies provide additional targets for manipulating both hematopoeitic and non-hematopoeitic cells involved in potentiating type 2 immune responses towards restoring the balance between tolerance and protective immunity at mucosal sites.

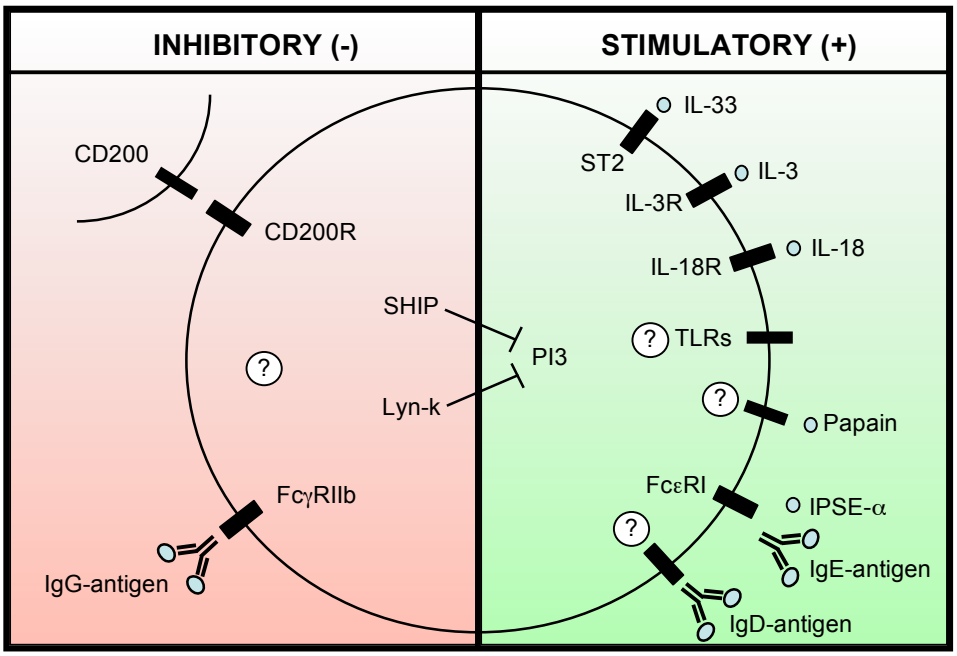

Figure 30. Multiple pathways of inhibiting and stimulating basophil effector functions.

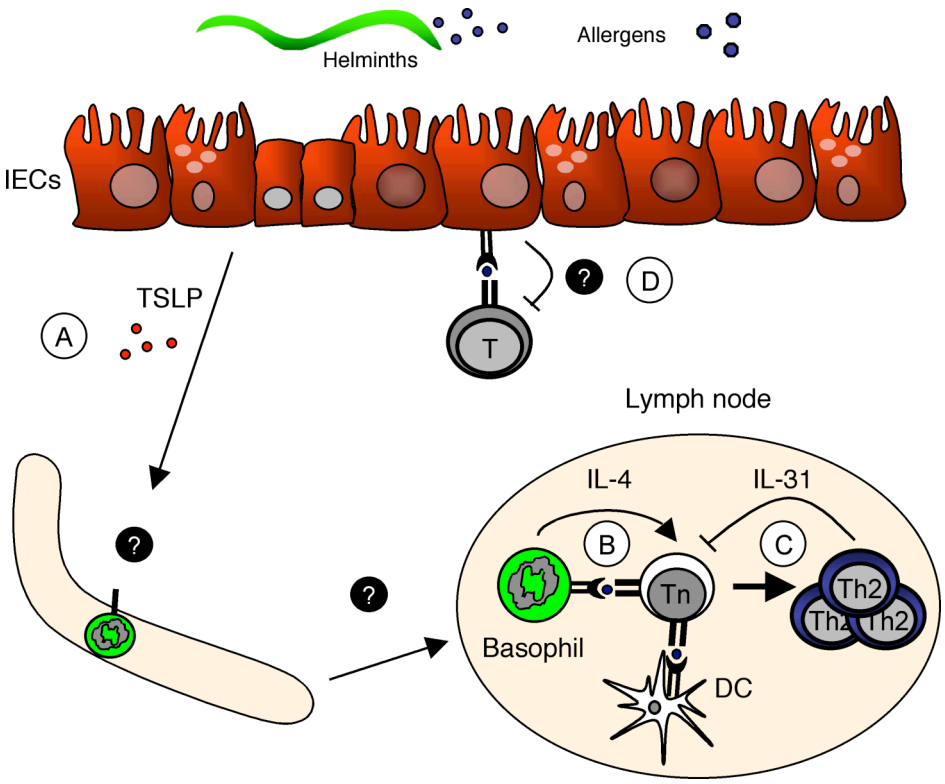

Figure 31. Thesis summary. A. Following exposure to helminth parasites or protease allergens, IECs are activated and produce cytokines including TSLP that can promote peripheral basophil responses. However whether TSLP acts directly on basophils is unknown. Basophils are also rapidly recruited to LNs through as yet uncharacterized mechanisms. B. In the LN, basophils expressing MHC class II and IL-4 may act in concert with DCs to promote Th2 cell differentiation. C. Th2 cells preferentially produce the cytokine IL-31 which may in turn negatively regulate Th2 cytokine production from naïve $CD4^+$ T cells through direct effects on T cells or indirectly through effects on accessory cells. D. Effector $CD4^+$ T cells transit to the lamina propria where through

unknown contact-dependent mechanisms, MHC class II-expressing IECs can inhibit their proliferation and cytokine production.

Appendix A

Intestinal epithelial cell-intrinsic MHC class II limits $CD4^+$ T cell-mediated intestinal inflammation during enteric infection

Abstract

Despite recent advances, the cellular requisites for the initiation and regulation of immune responses within the intestine remain poorly defined. Previous studies have shown that intestinal epithelial cells (IECs) play an active role in antigen sampling and possess the molecular machinery required for antigen processing and presentation via MHC class II. However, the functional consequences of antigen presentation by IEC are unknown. Here, we generate mice with a lineage-specific deletion of MHC class II within IEC (*iab*$^{\Delta IEC}$) to directly interrogate the functional consequences of IEC-intrinsic MHC class II expression on both tolerance and immunity within the gastrointestinal tract. While dispensable for the generation or maintenance of oral tolerance, MHC class II expression in IECs appears to be required to limit inflammation in a model of acute colitis using infection with the enteric pathogen *Citrobacter rodentium*. Following infection with *Citrobacter*, *iab*$^{\Delta IEC}$ mice exhibited increased expression of IFN-γ, TNFα and IL-17 and enhanced intestinal inflammation compared to their littermate controls and *in vitro*, IECs inhibited proliferation and cytokine production in activated $CD4^+$ T cells in a contact-dependent manner. Taken together, these results demonstrate a previously unrecognized role for cognate IEC-$CD4^+$ T cell interactions in limiting infection-induced intestinal inflammation.

Introduction

Early *in vitro* studies using epithelial cells isolated from rat and murine small intestine demonstrated that intestinal epithelial cells (IECs), while significantly less potent than professional antigen presenting cells, are capable of both processing and presenting antigen via the MHC class II pathway and may be a mechanism for antigen sampling and presentation in the intestine (Telega *et al.*, 2000; Kaiserlian, 1999; Kaiserlian *et al.*, 1989; Bland and Warren, 1986b; Bland and Warren, 1986a). Subsequent research utilizing human cell lines revealed that IECs constitutively express proteolytically active cathepsins B, L, and H and that IFN-γ stimulation results in the induced expression of the invariant chain and HLA-DM$\alpha\beta$ (Hershberg *et al.*, 1997). Together, these studies demonstrate that IECs express all the necessary components for MHC class II antigen processing and presentation and are capable of stimulating T cell proliferation *in vitro* but whether this occurs *in vivo* is unclear.

The functional role of IEC-intrinsic MHC class II expression is unknown and may differ depending on the location within the GI tract. For example, small intestinal epithelial cells constitutively express MHC class II in the absence of co-stimulatory molecules, suggesting a potential role for IEC-intrinsic MHC class II expression in the induction of oral tolerance. In contrast to the small intestine, the colonic epithelium in healthy individuals is largely MHC class II negative. However, in the context of inflammatory bowel disease or following exposure to pro-inflammatory cytokines such as IFN-γ, MHC class II expression in colonic IECs can be induced. In support of an *in vivo* role for MHC class II presentation by IECs, recent studies in Crohn's disease patients have shown that MHC class II expression is dramatically upregulated in IECs within diseased colonic

tissue compared to healthy tissue and that soluble ovalbumin (OVA) protein administered during biopsy is efficiently taken up by IECs and localized to late endosomes on their basolateral surface (Buning *et al.*, 2006). This has led to the hypothesis that while small intestinal epithelial cell expression of MHC class II may promote oral tolerance, the upregulation of MHC class II in the colon under inflammatory conditions may contribute to a breakdown in tolerance and enhanced activation of pro-inflammatory effector $CD4^+$ T cells.

Here we describe the generation of mice with an IEC-intrinsic deletion of MHC class II expression (*iab*$^{\Delta IEC}$ mice). Contrary to our prediction, loss of MHC class II within the intestinal epithelium did not affect the composition of lymphocytes within intestinal tissues, the proliferation of $CD4^+$ T cells in response to oral antigen or functional oral tolerance upon challenge with previously fed antigen. Deletion of IEC-intrinsic MHC class II expression did however influence the immune response to the enteric pathogen, *Citrobacter rodentium*. After infection with *Citrobacter*, *iab*$^{\Delta IEC}$ mice showed increased pro-inflammatory cytokine production and intestinal inflammation compared to *iab*$^{F/F}$ littermate controls, indicating a negative regulatory role for MHC class II-dependent IEC-$CD4^+$ T cell interactions. Additionally, co-culture of activated $CD4^+$ T cells with an immortalized MHC class II^+ colonic epithelial cell line led to significant decreases in T cell-derived IL-2 and TNFα, suggesting that IECs may directly limit cytokine production by $CD4^+$ T cells.

Materials and Methods

Mice and *Citrobacter rodentium* infection

C57BL/6 and Villin-Cre mice (B6.SJL-Tg(Vil-cre)997Gum/J) were ordered from The Jackson Laboratory. Mice with floxed alleles of IAb (*iab*$^{F/F}$ mice) were obtained from Pandelakis Koni (Medical College of Georgia). Mice with an IEC-intrinsic deletion of MHC class II expression (*iab*$^{\Delta IEC}$ mice) were generated by crossing Villin-Cre mice to *iab*$^{F/F}$ mice. F1 mice were then crossed and genotypes confirmed by Southern blot and PCR as previously described (Hashimoto *et al.*, 2002). MHC class II-eGFP reporter mice (*iab-egfp*) were generated by Hidde Ploegh (Boes *et al.*, 2002) and provided by Jatin Vyas (The Whitehead Institute for Biomedical Research). All experiments were performed following the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. *Citrobacter rodentium* was a gift from Bruce Valence (University of British Columbia). For infections, an isolated colony of *Citrobacter* was grown overnight in 5ml of Luria Broth and mice infected by oral gavage with 200μl of culture. 10μl of the overnight culture was streaked onto selective MacConkey agar to determine infective dose in colony forming units (CFU) per ml (between $1x10^8$-$1x10^9$ CFU per infection). For colonic explant cultures, an ~0.5cm section of tissue was isolated from the distal colon and cut open longitudinally, immediately placed in complete tissue culture media and cultured for 24 hours at 37 degrees C. Tissue was removed the next day, blotted on paper towel and weighed. Cytokines were measured by ELISA and normalized to the weight of the colonic tissue.

Isolation and flow cytometry of primary IEC

The small intestine, large intestine and ceca from naïve or infected mice were removed and cut open longitudinally using blunt-end scissors. The tissue was washed vigorously in pre-warmed 37°C PBS twice to remove fecal matter, placed in 10 mls pre-warmed stripping media (PBS, 1mM EDTA, 1mM DTT, 5% FBS and 1X penicillin/streptomycin) and shaken at 37°C for 10 min. Following the 10 min strip, the tissue was allowed to settle briefly and IEC-containing supernatant passed through a 70um cell strainer, pelleted at 1200 RPM for 5 min, re-suspended in complete media and placed on ice. IEC were then stained with fluorochrome-conjugated antibodies against epithelial adhesion molecule (EpCam; clone G8.8) and MHC class II IA/IE (clone M5114) and stained for viability with TOPRO3 immediately before flow cytometric analysis on a FACSCalibur.

***In vivo* T cell proliferation assay**

$CD4^+$ OTII CD45.1 T cells were isolated from spleens and lymph nodes either by negative selection via incubation with hybridoma supernatants (αB220, $\alpha F_C R$, αCD8, αMHCII) followed by magnetic bead purification (Qiagen). Purified $CD4^+$ T cells were re-suspended in PBS, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) for 5 min and the labeling quenched with FBS. 1 million CFSE-labeled $CD4^+$ OTII CD45.1 T cells were transferred i.v. to recipient $iab^{\Delta IEC}$ and $iab^{F/F}$ mice on day -1. Ovalbumin (OVA) was added to the drinking water at a concentration of 1.5g/L at day 0 and mice were sacrificed at day 4. Mesenteric lymph nodes were harvested, stained with fluorochrome-conjugated antibodies against CD4, CD45.1 and CD45.2 and analyzed by flow cytometry.

Oral tolerance model

iab$^{\Delta IEC}$ and *iab*$^{F/F}$ mice were given either normal drinking water or drinking water supplemented with 1.5g/L OVA for 5 days. On day 5, mice were sensitized with 100ug OVA in IFA in the footpad followed two weeks later by a challenge with OVA/IFA in the contralteral footpad. On day 2 after the challenge, popliteal lymph nodes were harvested, plated in a 96-well plate and re-stimulated with OVA for 48 hours and supernatants analyzed by ELISA for IFN-γ. Serum was analyzed by standard sandwich ELISA for OVA-specific Ig.

ImmortoIEC culture

The immortalized colonic epithelial cell line (ImmortoIEC) was maintained in Ham's F-12 media (Cellgro) supplemented with 10% heat-inactivated FBS, 2 nM L-glutamine, 100 U penicillin G, 100 ug/ml streptomycin, Insulin Transferrin Selenium (Gibco), and 40 ng/ml murine IFN-γ (RnD) in a humidified incubator with 5% CO_2 at 37°C.

ImmortoIEC:CD4$^+$ T cell co-culture

CD4$^+$ T cells cells were isolated from spleens and lymph nodes via negative selection by incubation with hybridoma supernatants [anti-B220 (RAE), anti-FcR (24G2), anti-CD8 (2.43) and anti–MHC class II (M5114)] followed by magnetic bead purification (Qiagen) or by fluorescence activated cell sorting (FACS). OT-II cells were labeled with CFSE and seeded at a density of 5 x 10^5 per ml with or without 7.5 x 10^4 per ml ImmortoIECs in anti-CD3ε-coated (145-2C11; eBioscience) 96-well plates with soluble OVA peptide (1 mg/ml) and anti-CD28 (37.51; eBioscience). For transwell assays, cells were cultured in 24-well plates with out without separation by a transwell (BD; cat. # 353104). After 4 d of culture, cells were stimulated for 4 h with phorbol 12-myristate 13-acetate, ionomycin

and brefeldin A. Cells were pelleted for 5 min at 485g. Supernatants were collected for ELISA and cells were washed in flow cytometry buffer, stained with fluorochrome-conjugated monoclonal anti-CD3ε (17A2; eBioscience) and anti-CD4 (RM4-5; eBioscience) and fixed with 2% (vol/vol) paraformaldehyde. Cells were permeablized with 0.5% (wt/vol) saponin in flow cytometry buffer and stained for intracellular cytokines with fluorochrome-conjugated monoclonal anti-IL-2 (JES6-5H4; BD Biosciences) and TNFα. Standard sandwich ELISAs were performed using commercially available antibody pairs (eBioscience).

Results

Generation and characterization of iab$^{\Delta IEC}$ mice

To determine the biological functions of IEC-intrinsic MHC class II expression, mice with *loxP* sites flanking the promoter region and exon 1 of IA^b β-chain alleles (Hashimoto *et al.*, 2002) (*iab*$^{F/F}$) were crossed to mice expressing Cre recombinase under control of the IEC-specific villin promoter (Vil-Cre) to generate mice with a lineage-specific deletion of MHC class II in IECs (*iab*$^{\Delta IEC}$). *iab*$^{\Delta IEC}$ mice were born in expected Mendelian ratios and exhibited normal frequencies of lymphoid cells in the spleen, peripheral lymph nodes and intestinal tissue (data not shown). Specific deletion of MHC class II in IECs was confirmed by flow cytometry of gut associated lymphoid tissue and stripped small intestinal epithelium of *iab*$^{\Delta IEC}$ mice and *iab*$^{F/F}$ littermate control mice. Conventional antigen presenting cells isolated from Peyer's patches including B cells and dendritic cells were MHC class II-positive in both *iab*$^{\Delta IEC}$ mice and *iab*$^{F/F}$ littermate control mice (**Fig. 32a**). In contrast, IEC-intrinsic expression of MHC class II was efficiently deleted within the small intestine of *iab*$^{\Delta IEC}$ mice but not *iab*$^{F/F}$ littermate control mice (**Fig. 32b**).

Importantly, T cell development in the thymus was unaffected by deletion of MHC class II in IECs (**Fig. 32c**) and normal ratios of $CD4^+$ and $CD8^+$ T cells were observed in peripheral lymphoid organs (**Fig. 33**). Adoptive T cell transfer experiments into MHC class II-deficient hosts have implicated MHC class II-dependent interactions in maintaining homeostasis of $CD4^+$ T cells in the periphery and a previous study utilizing mice with a conditional deletion of MHC class II in hematopoietic and endothelial cells revealed a significant decrease in total $CD4^+$ T cell numbers (Shimoda *et al.*, 2006). We therefore hypothesized that IEC-intrinsic MHC class II expression may influence the retention of $CD4^+$ T cells within the GALT, including intestinal intraepithelial (IEL) and lamina propria lymphocytes (LPL). To test this, we isolated the spleen, Peyer's patches, mLNs and small and large intestine IELs and LPLs from both $iab^{\Delta IEC}$ mice and $iab^{F/F}$ littermate control mice and analyzed $CD4^+$ T cells by flow cytometry. The frequency (**Fig. 32d**) and number (data not shown) of $CD4^+$ T cells within all intestinal tissues evaluated was equivalent between $iab^{\Delta IEC}$ mice and $iab^{F/F}$ littermate control mice suggesting no inherent defect in intestinal $CD4^+$ T cell homeostasis in the absence of IEC-intrinsic MHC class II expression. Additionally, gross pathological analyses revealed no signs of spontaneous intestinal inflammation between $iab^{\Delta IEC}$ mice and $iab^{F/F}$ littermate control mice (**Fig. 32e**).

Intact oral tolerance in $iab^{\Delta IEC}$ mice

The constitutive expression of MHC class II within the small intestinal epithelium in the absence of high levels of co-stimulatory molecule expression has led to the hypothesis that IEC-intrinsic MHC class II expression may promote tolerance to luminal antigens including food and commensal-derived products (Sanderson *et al.*, 1993). We therefore

sought to determine whether the $CD4^+$ T cell response to orally-delivered antigen was perturbed in $iab^{\Delta IEC}$ mice utilizing an antigen-specific feeding model. Previous studies have demonstrated that following oral exposure to ovalbumin (OVA), antigen-specific $CD4^+$ T cells proliferate and acquire a regulatory phenotype (Coombes *et al.*, 2007; Sun *et al.*, 2007). To test whether IEC-intrinsic MHC class II expression was required for the proliferation of $CD4^+$ T cells in response to oral antigen, CFSE-labeled OVA-specific OTII $CD4^+$ T cells were adoptively transferred into $iab^{F/F}$ and $iab^{\Delta IEC}$ mice that were then given OVA in their drinking water for four days. OTII cells from the mesenteric lymph nodes of $iab^{F/F}$ and $iab^{\Delta IEC}$ mice given normal drinking water showed minimal proliferation as measured by CFSE dilution (**Fig 34a**). In contrast, in response to orally administered OVA, OTII $CD4^+$ T cells from both $iab^{F/F}$ and $iab^{\Delta IEC}$ mice exhibited extensive and equivalent rounds of proliferation indicating there was no defect in the induction of $CD4^+$ T cell proliferation in response to oral antigen in the absence of IEC-intrinsic MHC class II (**Fig. 34a**). Consistent with these data, we did not detect any differences in the frequencies of $Foxp3^+$ T_{regs} between $iab^{F/F}$ and $iab^{\Delta IEC}$ mice in secondary lymphoid organs and gut-associated lymphoid tissues (**Fig. 33b**).

To determine whether functional oral tolerance was intact in $iab^{\Delta IEC}$ mice, $iab^{F/F}$ littermate control and $iab^{\Delta IEC}$ mice were given either normal drinking water or drinking water containing OVA for four days followed by an OVA sensitization and subsequent OVA challenge. Mice that received normal drinking water developed robust OVA-specific IFN-γ responses (**Fig. 34b**) and Ig titers (**Fig. 34c**). However, both $iab^{F/F}$ and $iab^{\Delta IEC}$ mice fed OVA prior to sensitization and challenge remained non-responsive compared to unfed controls (**Fig. 34b,c**). Collectively, these data suggest that MHC class II

expression within the intestinal epithelium is not essential for the generation of functional oral tolerance.

Exacerbated inflammation following *Citrobacter rodentium* infection in the absence of IEC-intrinisc MHC class II expression

An alternative function for antigen presentation by IECs could be in the initation or regulation of $CD4^+$ T cell responses to enteric pathogens. Previous studies have shown that exposure to pro-inflammatory cytokines such as IFN-γ is associated with the up-regulation of MHC class II within the colonic epithelium and we hypothesized that the inflammation following acute enteric infection may also induce colonic IEC to express MHC class II. To test this, we employed infection with the attaching-effacing bacterium *Citrobacter rodentium* which results in an acute colitis associated with epithelial cell hyperproliferation, crypt elongation and loss of goblet cells analgous to enteropathogenic *E. coli* (EPEC) infection in humans (Higgins *et al.*, 1999). During the course of infection, *Citrobacter* is closely associated with IEC, altering the actin cytoskeleton to form a pedestal-like structure on the apical cell surface. Clearance of *Citrobacter* is critically dependent on $CD4^+$ T cells, B cells and the cytokines IFN-γ, IL-23 and IL-22 (Zheng *et al.*, 2008; Mangan *et al.*, 2006; Mundy *et al.*, 2005; Simmons *et al.*, 2003). The intimate association of *Citrobacter* with colonic IECs, coupled with the requirement for $CD4^+$ T cells in antibacterial immunity, makes it a tractable model to study the role of IEC-intrinsic MHC class II during enteric infection.

We first examined whether *Citrobacter* infection could induce MHC class II expression in colonic IEC using MHC class II-eGFP (*iab-egfp*) reporter mice (Boes *et al.*, 2002). The distal colon was isolated from naïve and infected *iab-egfp* mice and imaged using 2-

photon microscopy. While MHC class II expression was limited to what were likely dendritic cells within the lamina propria and IELs, following infection there was detectable MHC class II expression within epithelial cells that appeared to increase in intensity near the base of the crypt (**Fig. 35a, arrows**). To obtain a quantitative measure of the frequency of MHC class II^+ epithelial cells within the colon, IECs were stripped from colonic tissue isolated from naïve and infected WT mice and analyzed by flow cytometry. Compared to naïve mice, *Citrobacter* infection induced a two-fold increase in the frequency of MHC class II^+ IEC (**Fig. 35b**).

To determine whether induction of MHC class II on IEC had functional consequences for enteric immunity, we infected $iab^{F/F}$ littermate control and $iab^{\Delta IEC}$ mice with *Citrobacter*. The lack of MHC class II on IEC did not alter the ability of *Citrobacter* to establish infection as determined by colony forming units (CFU) per gram of stool (**Fig. 36a**). However, despite equivalent bacterial burdens at day ten post-infection, $iab^{\Delta IEC}$ mice exhibited exacerbated pro-inflammatory cytokine responses compared to their $iab^{F/F}$ littermate controls. In colon explant cultures, that measure spontaneous cytokine production directly ex vivo, $iab^{F/F}$ mice exhibited infection-induced increases in production of IFN-γ, TNF-α and IL-10 (**Fig. 36b**). While infection-induced IL-10 was equivalent between $iab^{F/F}$ littermate control and $iab^{\Delta IEC}$ mice, $iab^{\Delta IEC}$ mice had elevated expression of the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-17 (**Fig. 36b**). Additionally, $CD4^+$ T cells isolated from the colonic lamina propria of $iab^{\Delta IEC}$ mice produced elevated levels of infection-induced IL-17 compared to littermate control mice by intracellular cytokine staining (**Fig. 36c**). Following infection, histological analysis of the distal colon of $iab^{F/F}$ mice showed characteristic crypt hyperplasia, loss of goblet cells

and signs of inflammatory infiltrate (**Fig. 36d**). Consistent with the observed increases in pro-inflammatory cytokine production, distal colon from infected *iab*$^{\Delta IEC}$ mice showed enhanced inflammation characterized by increased aggregates of neutrophils and apoptotic cells (**Fig. 36d**). Taken together, these data suggest that IEC-intrinsic MHC class II expression is important for regulating infection-induced inflammation in the large intestine.

IECs inhibit CD4$^+$ T cell proliferation and cytokine production

Previous studies have suggested that IECs may regulate CD4$^+$ T cell proliferation and cytokine expression (Cruickshank *et al.*, 2004; Yamamoto *et al.*, 1998). We therefore hypothesized that MHC II expression on IECs could facilitate the immune regulation of antigen-specific CD4$^+$ T cells and that the elevated Type 17 inflammatory response in *iab*$^{\Delta IEC}$ mice during *Citrobacter* infection could be due the absence of IEC-derived contact-dependent immunoregulatory signals.

To investigate whether IECs have a regulatory role in CD4$^+$ T cell activation and cytokine production, we employed an *in vitro* co-culture system with CD4$^+$ T cells and a colonic epithelial cell line (ImmortoIEC). The ImmortoIEC line used in this system was derived from the Immortomouse (Boes *et al.*, 2002) that contains a temperature-sensitive version of the oncogene SV40 that is stable at 33°C, but not 37°C, allowing for the efficient generation of a cell line from the tissue of interest. We first examined whether ImmortoIECs could regulate CD4$^+$ T cell proliferation and cytokine expression in the presence of polyclonal anti-CD3/CD28 stimulation. After 4 days of culture without ImmortoIECs, most OT-II CD4$^+$ T cells had undergone proliferation, as measured by CFSE-dilution, and expressed IL-2 and TNF-α (**Fig. 37a**). In contrast, addition of

ImmortoIECs led to reduced $CD4^+$ T cell proliferation and a decrease in the frequency of $CFSE^{dim}$ OT-II cells expressing IL-2 and TNF-α (**Fig. 37a**). The decrease in IL-2 and TNF-α expression correlated with a decrease in cytokine detected by ELISA (**Fig. 37b**).

To determine whether the negative regulation of $CD4^+$ T cell proliferation and cytokine expression by ImmortoIECs was mediated by soluble factors or by cell-cell contact, we repeated the co-culture in the presence or absence of a transwell separating the OT-II $CD4^+$ T cells and the ImmortoIECs. Consistent with our previous results, addition of ImmortoIECs in contact with OT-II $CD4^+$ T cells led to a reduction of total $CFSE^{dim}$ cells as well as the frequency of $CFSE^{dim}$ cells expressing IL-2 and TNF-α when compared to OT-II $CD4^+$ T cells cultured alone. (**Fig. 37c**). However, separation of the ImmortoIECs from the OT-II $CD4^+$ T cells by a transwell reversed the negative regulation of proliferation and IL-2 and TNF-α expression (**Fig. 37c**). The effects of IEC interactions on $CD4^+$ T cell intracellular cytokine production also correlated with the amount of secreted IL-2 and TNF-α detected by ELISA (**Fig. 37d**). These data suggest that intestinal epithelial cells can regulate the proliferation and cytokine expression of activated $CD4^+$ T cells and that this regulation is mediated via cell-cell contact.

Discussion

While MHC class II expression within the intestinal epithelium was first reported over 25 years ago, the biological function of MHC class II expression on non-hematopoeitic cells such as IECs has remained unclear. In this study, we directly interrogated this question through the generation of mice with a lineage-specific deletion of MHC class II within IECs ($iab^{\Delta IEC}$ mice). As interactions between the TCR and MHC have been reported to

prolong the lifespan of $CD4^+$ T cells (Brocker, 1997; Kirberg *et al.*, 1997; Takeda *et al.*, 1996) we hypothesized that, if IECs are a major source of intestinal MHC II, loss of IEC-intrinsic MHC II could lead to reduced survival or retention of $CD4^+$ T cells in the gut. However, deletion of MHC II in IECs did not alter the size of the $CD4^+$ T cell population in either peripheral lymphoid organs or intestinal tissues indicating that IEC-derived MHC II does not play a critical role in maintaining the intestinal $CD4^+$ T cell homeostasis.

Another proposed function for IEC-intrinsic MHC class II expression is in the generation or maintenance of oral tolerance. Previously published studies have demonstrated that IECs secrete 30-90nm diameter vesicles named exosomes that are decorated with antigen-loaded MHC II and the putative basolateral secretion of these exosomes could influence the induction of immunity or tolerance to orally delivered antigens (Mallegol *et al.*, 2005; van Niel *et al.*, 2001). One such study termed these exosomes "tolerosomes" as purified IEC-derived exosomes were able to promote tolerance when transferred to naïve hosts (Karlsson *et al.*, 2001). Alternativley, as IECs are thought to express low levels of co-stimulatory molecules (Sanderson *et al.*, 1993), direct MHC class II-TCR interactions between IECs and T cells could result in a classical "signal 1" without "signal 2" form of T cell tolerization (Kapsenberg, 2003). Current models of oral tolerance include priming of T cells by tolerogenic $CD103^+$ dendritic cells (DCs) present within intestinal tissues that can promote peripheral Treg conversion through a retinoic acid- and TGFβ-dependent mechanism (Coombes *et al.*, 2007; Sun *et al.*, 2007). Here, we demonstrate that the loss of IEC-intrinsic MHC II impairs neither the priming of $CD4^+$ T cells to oral antigen nor the generation of functional oral tolerance. It therefore remains unclear what function constitutive expression of IEC-intrinsic MHC class II serves in the

small intestine of a naïve host. However, while we have demonstrated that IEC-derived antigen-MHC II complexes are not required for the generation of oral tolerance, it remains possible that tolerogenic $CD103^+$ DCs and IECs play redundant roles in these biological processes.

The inducible expression of MHC class II within the large intestine via exposure to IFN-γ (Buning *et al.*, 2005) or as a result of enteric infection in mouse models (Higgins *et al.*, 1999) coupled with the selective expression of MHC class II within inflamed versus healthy tissue in human IBD patients (Buning *et al.*, 2006) has prompted the opposing hypotheses that inducible IEC-intrinsic MHC II may function to either exacerbate or alternatively to restrain intestinal inflammation. The primary function of IECs in intestinal immunity has been as a physical barrier between the luminal contents of the gut and the underlying lamina propria lymphocytes. However, recent reports suggest that IECs play a more active role in regulating intestinal immune responses from conditioning DCs to promote tolerogenic T cell responses (Rimoldi *et al.*, 2005) to influencing B cell IgA isotype switching (Cerutti, 2008). Two previously published studies also support a role for IEC-intrinsic NFκB activation in limiting spontaneous or infection-induced intestinal inflammation (Nenci *et al.*, 2007; Zaph *et al.*, 2007). Thus, an important role for IECs beyond maintaining the epithelial barrier is emerging, whereby IECs actively regulate intestinal immune responses.

Here, we report a novel MHC II-dependent role for IECs in limiting intestinal inflammation *in vivo*. We find that genetic deletion of IEC-intrinsic MHC class II leads to an increase in inflammatory cytokine production in intestinal tissue, infiltration of IL-17-

expressing $CD4^+$ T cells into the lamina propria, and exacerbated intestinal inflammation after *Citrobacter* infection. Based on previous studies (Yamamoto *et al.*, 1998), we hypothesized that MHC II expression on IECs could facilitate immune regulation of antigen-specific $CD4^+$ T cells. Lack of MHC class II on IECs would therefore remove an immunoregulatory arm of the *Citrobacter*-specific response, leading to increased inflammatory cytokine production.

Consistent with the *in vivo* regulation of $CD4^+$ T cells by IECs, activation of $CD4^+$ T cells in the presence of ImmortoIECs *in vitro* led to a negative regulation of both proliferation and cytokine expression. Separation of ImmortoIECs from $CD4^+$ T cells by a trans-well abrogated the negative regulation of both proliferation and cytokine expression, consistent with a contact-dependent mechanism for IEC regulation of $CD4^+$ T cells. We propose that formation of an "effector immunological synapse" between colonic epithelial cells and activated antigen-specific $CD4^+$ T cells within the IEL and lamina propria allows for the transmission of contact-dependent immuno-regulatory signals that limit pro-inflammatory cytokine production during enteric infection. Future work exploring the mechanisms responsible for this IEC-mediated immune regulation may lead to novel effective therapies for mucosal inflammatory diseases including colitis and food allergy.

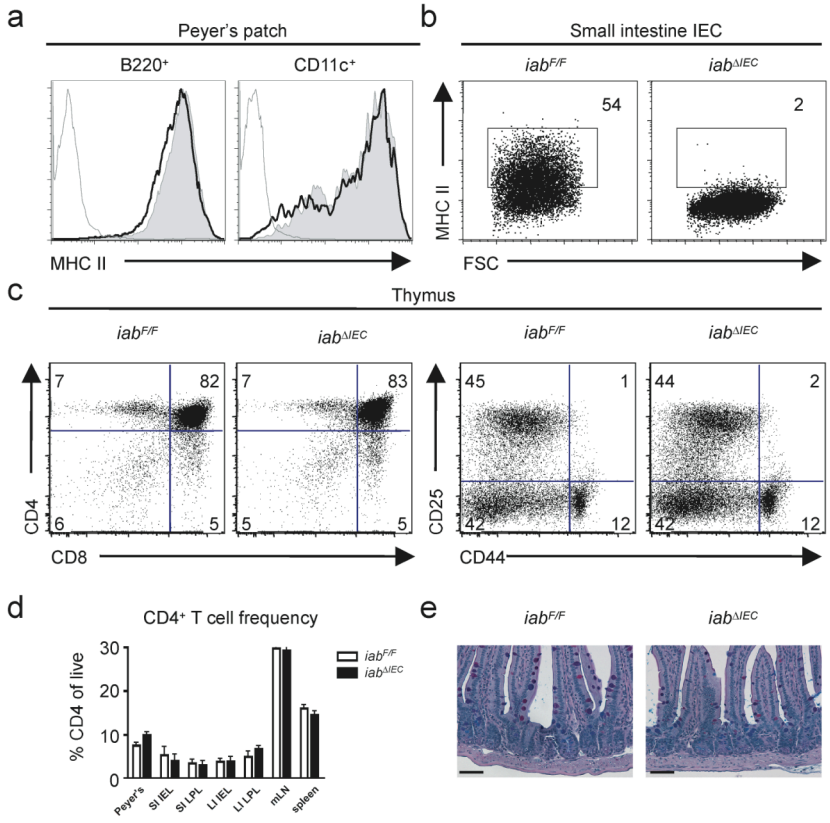

Figure 32. Generation and characterization of *iab*$^{\Delta IEC}$ mice. (a) Histogram of Peyer's patch cells stained with antibodies against B220, CD11c and MHC class II. Grey line = isotype control, shaded histogram = *iab*$^{F/F}$ mice and black line = *iab*$^{\Delta IEC}$ mice. (b) Flow cytometry of stripped epithelial cells from the small intestine of *iab*$^{F/F}$ and *iab*$^{\Delta IEC}$ mice stained for MHC class II; plots gated on EpCam^{pos}, TOPRO^{neg} cells. Numbers in plots represent frequency of cells within gated area. (c) Flow cytometry of thymocytes from *iab*$^{F/F}$ and *iab*$^{\Delta IEC}$ mice stained with antibodies against CD8, CD4, CD44 and CD25. Numbers in plots represent frequency of cells within quadrant. (d) Frequencies of CD4^{+} T cells within intestinal tissues and spleens of *iab*$^{F/F}$ and *iab*$^{\Delta IEC}$ mice determined by flow

cytometry. Open bars = *iab*$^{F/F}$ mice; filled bars = *iab*$^{\Delta IEC}$ mice. (e) Alcian blue/Periodic Acid Schiff's staining of small intestinal tissue isolated from *iab*$^{F/F}$ and *iab*$^{\Delta IEC}$ mice. Bar = 20μm.

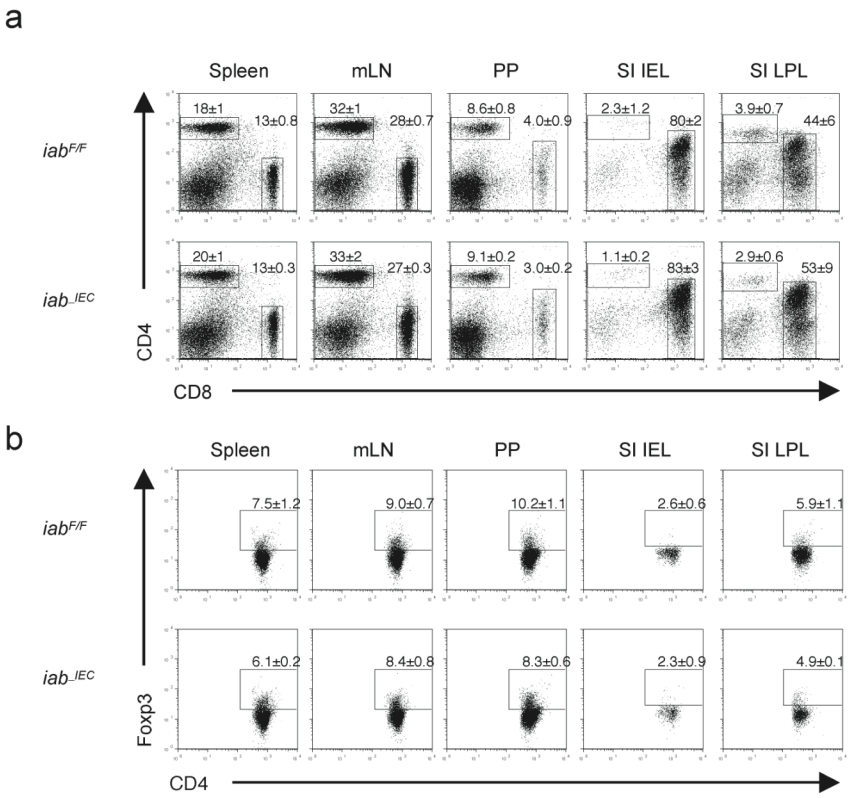

Figure 33. iab$^{F/F}$ and iab$^{\Delta IEC}$ exhibit similar frequencies of T cells subsets. Flow cytometry of spleen, mesenteric lymph node (mLN), peyer's patches (PP), and small intestine (SI) intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) from *iab$^{F/F}$* and *iab$^{\Delta IEC}$* mice stained with antibodies against CD3, CD8, CD4 and Foxp3.

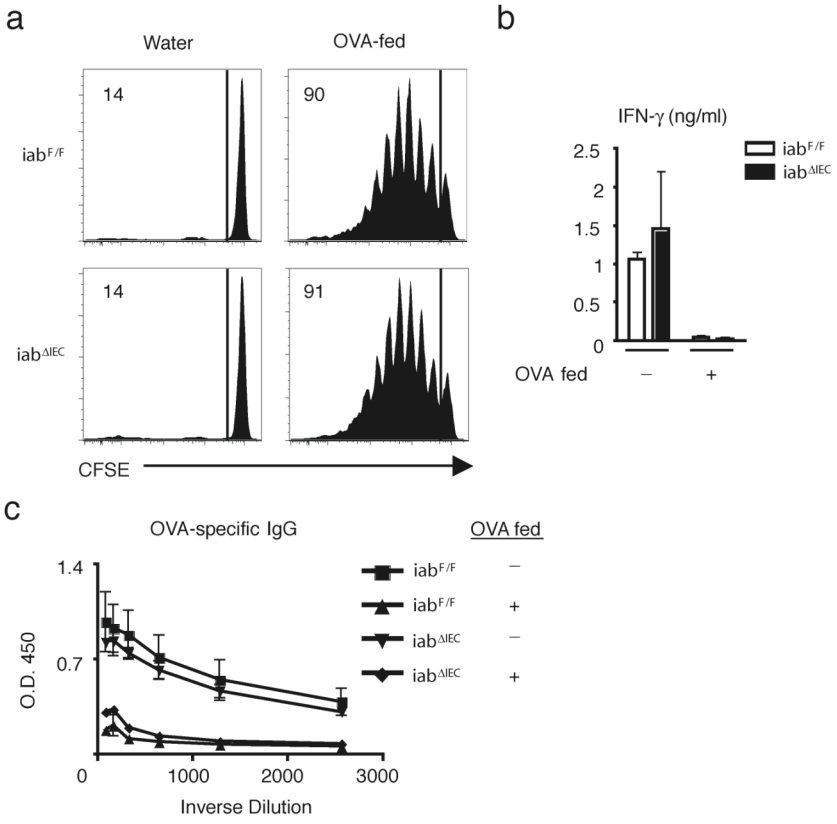

Figure 34. Oral tolerance is intact in *iab*$^{\Delta IEC}$ mice. (a) Histograms of CFSE-labeled OVA-specific OTII CD4$^+$ T cells transferred into either *iab*$^{F/F}$ or *iab*$^{\Delta IEC}$ mice that were given normal water or water with 1.5mg/ml OVA protein for four days. Plots gated on CD45.1$^+$ CD4$^+$ cells; numbers in plots represent frequency of CFSE-dim cells. (b) IFN-γ secretion by popliteal LN cells from OVA-challenged *iab*$^{F/F}$ and *iab*$^{\Delta IEC}$ mice given either normal water or water with 1.5μg/ml OVA protein for four days. pLN cells were harvested two days after challenge, re-stimulated with OVA for 48 hours and IFN-γ in supernatants measured by ELISA. (c) OVA-specific IgG levels in serum from mice treated as in (b).

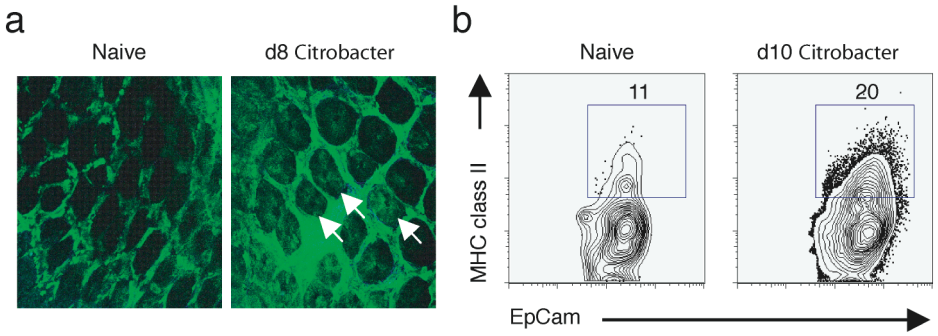

Figure 35. IEC upregulate MHC class II expression following *Citrobacter* infection. (a) Two-photon microscopy of the large intestine of naïve and day 8 *Citrobacter*-infected MHC class II-eGFP reporter mice. Arrows indicate MHC class II-eGFP^{+} IEC. (b) Flow cytometry of stripped large IEC from naïve or infected WT C57BL/6 mice stained for MHC class II and EpCam.

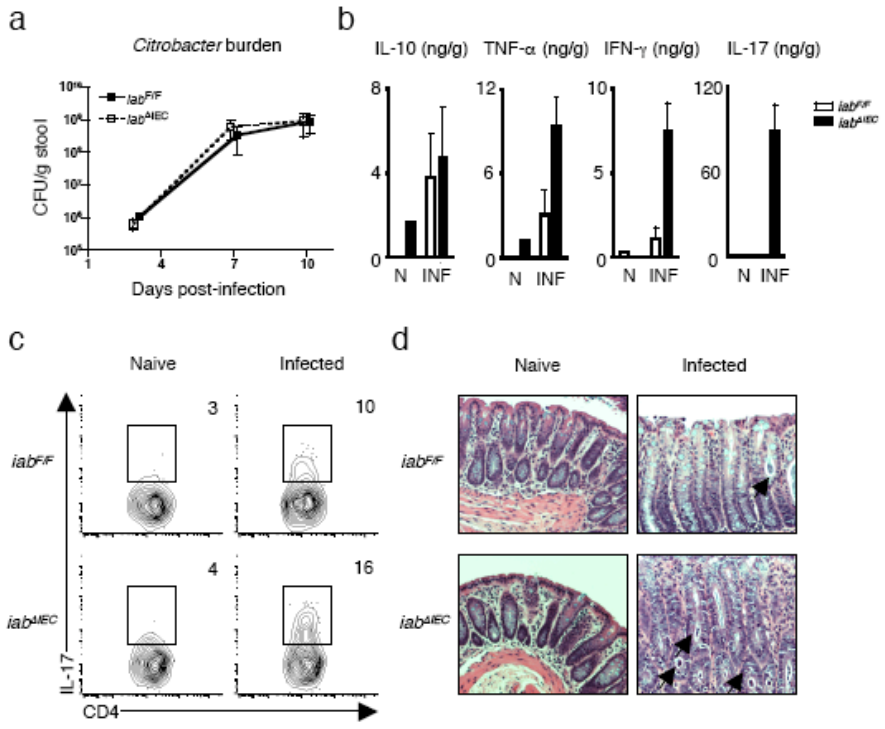

Figure 36. Exacerbated infection-induced inflammation in the absence of IEC-intrinsic MHC class II. (a) *Citrobacter* burden in colony forming units (CFU) per gram of feces determined by growth on selective media. (b) ELISAs from ex-vivo organ explant culture. Sections of distal colon from naïve and infected *iab*$^{F/F}$ and *iab*$^{\Delta IEC}$ mice were cultured for 24 hours and supernatants analyzed for spontaneous IL-10, IFN-γ, TNFα and IL-17 cytokine secretion. Open bar = *iab*$^{F/F}$; filled bar = *iab*$^{\Delta IEC}$. (c) Intracellular IL-17 staining of lamina propria CD4^{+} T cells isolated from naïve and infected *iab*$^{F/F}$ and *iab*$^{\Delta IEC}$ mice. Plots gated on CD4^{+} T cells; numbers indicate

frequency of cells within IL-17^{+} gate. (d) H&E staining of distal colon sections from naïve and infected *iab*$^{F/F}$ and *iab*$^{\Delta IEC}$ mice. Arrows indicate abscessed crypts.

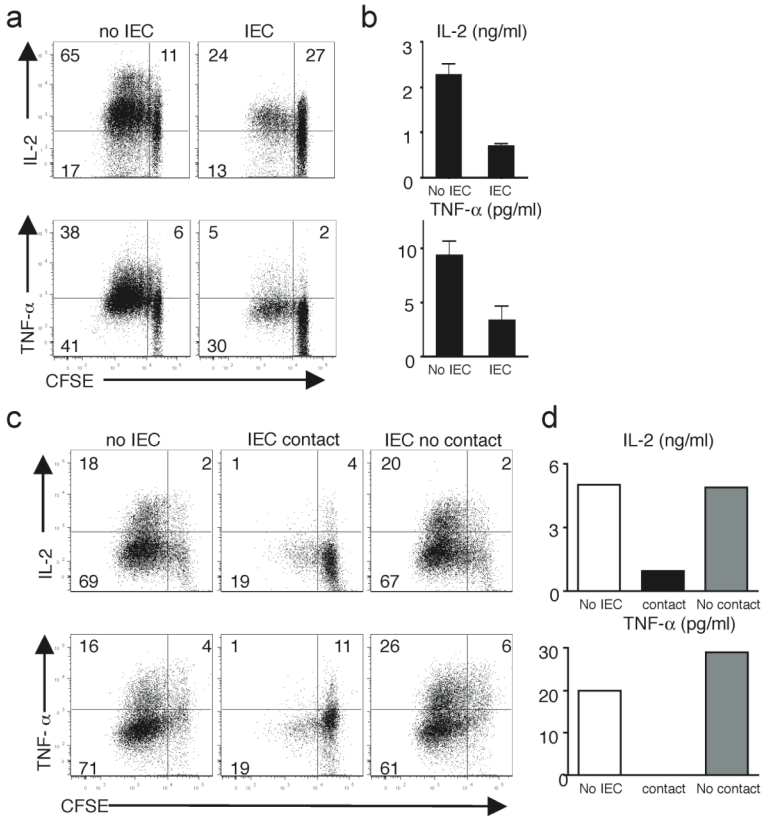

Figure 37. IECs inhibit cytokine production from T cells in a contact-dependent manner. (a) Flow cytometry of CFSE-labeled OT-II $CD4^+$ T cells simulated for 4 days with anti-CD3/CD28 in the presence or absence of ImmortoIECs. Numbers in plots represent frequency of cells within quadrant. (b) Supernatants from (a) were analyzed for IL-2 and TNF-α. (c) Flow cytometry of CFSE-labeled OT-II $CD4^+$ T cells that were cultured for 4 days with poly-clonal anti-CD3/CD28 stimulation in the presence or absence of ImmortoIECs in the well or separated by a transwell. Numbers in plots represent frequency of cells within quadrant. (d) Supernatants from (c) were analyzed for IL-2 and TNF-α cytokine secretion by standard sandwhich ELISA.

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